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Analysis of cumulative effects of multiple stressors on saltmarshes and consideration of management options

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Abstract

In addition to pressures exerted by natural physical-chemical forcings, natural systems are currently faced with a myriad of co-occurring human stressors as a result of economic and social drivers. There is evidence that stressors interact to cause larger than expected effects, thereby presenting a challenge to ecosystem management. This thesis aimed to develop new information that can contribute to reduce the existing knowledge gaps hampering the holistic management of multiple stressors. This was done via integrating reviews, field experiments and spatial assessment using North Adriatic saltmarshes as model system. I first undertook a review of the state-of-the-art methods to detect, quantify and predict stressor interactions, identifying techniques that could be applied this thesis research. Next, I conducted a systematic review of saltmarsh multiple stressor studies in conjunction with a multiple stressor mapping exercise for the study system in order to infer potential important synergistic stressor interactions. This analysis identified key stressors that currently affect the study system, but also pointed to large gaps in terms of driver and pressure data and raised issues for potentially overlooked stressors in the study system. Using field mesocosms, I explored how a local stressor (nutrient availability) affects the responses of vegetation to a global stressor (increased inundation) in different soil types. Results showed that saltmarsh vegetation would be more drastically affected by increased inundation in low than in medium organic matter soils, and especially in estuaries already under high nutrient availability. I conducted another field study examining the challenges of managing co-occurring and potentially interacting local stressors on saltmarsh vegetation: recreational trampling and smothering by deposition of excess macroalgal wrack due to high nutrient loads. Trampling and wrack prevention were found to have interacting effects, causing non-linear responses of the vegetation to simulated management of these stressors; vegetation recovered only in those treatments simulating the combined removal of both stressors. When either trampling or wrack smothering was removed individually, no significant benefits were observed. Finally, I carried out extensive mapping to analyze the rapid displacement of native *Spartina maritima* by cryptic, non-indigenous *Spartina* in north Adriatic saltmarshes. *S. anglica* was found to be unexpectedly widespread in the area, being well established virtually along the whole study region. Some samples of *S. townsendii* were also found. These non-indigenous species were nearly indistinguishable from the native species in growth form or patterns of spatial distribution. These previously undocumented stressors could pose additional challenges to the conservation of these fragile and highly threatened systems.

Acknowledgements

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As ecologists, much of our knowledge is gained in the field and having access to good study sites is of utmost importance. Therefore, I would like to thank the following organizations for their help in securing access to the study sites used in this thesis: the Corpo Forestale dello Stato (Ufficio territoriale per la Biodiversità), Camping Lido di Spina (Bellocchio), Azienda Agricola e Valliva Vallona e Santa Margherita di Antonio Bertaglia and Servizio Forestale Regionale della Regione del Veneto.

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Table of Contents

Chapter 1 Introduction

There is ample scientific evidence showing that stressors frequently co-occur, and when examined in experimental settings, often interact to result in unexpected effects on receptors (Crain, Kroeker & Halpern 2008; Przeslawski, Byrne & Mellin 2014; Strain *et al.* 2014). However, management processes up until recently was strongly focused on working in an impact-by-impact framework, and there are deeply-rooted scientific, cultural and institutional challenges to shifting those processes towards ecosystem-based management (Crowder *et al.* 2006). A large number of decision making tools are being developed to assess the tradeoffs associated with alternative management choices and to identify the best management option in a multiple stressor setting (e.g. InVEST: Integrated Valuation of Environmental Services and Tradeoffs). However, the use of these tools is often hampered by lack of habitat and pressure data which limits their current potential. Although legislation increasingly recognizes the importance of and requires the assessment of cumulative stressors for strategic or regional planning purposes (e.g. EU Marine Strategy Directive (2008/56/EC)), stressor effects are still often assumed to be additive as a consequence of lack of empirical knowledge of when, where and how stressor interactions occur (Halpern & Fujita 2013).

Challenges of managing coastal zones are particularly large. The world's coastal zones support about 60% of human population and the myriad of economic and social activities carried out here result in multiple stressors on the natural system, in addition to strong abiotic stress gradients related to the transition between land and sea (Harley 2003). Growing global populations and rising consumption rates are leading to increased resource extraction and polluting activities (e.g. fisheries, conversion of coastal habitats for agriculture and urban expansion, mining etc.), whilst unavoidable climate change is expected to put additional pressure on already stressed coastal systems (Wong *et al.* 2014). In order to avoid costly ecological surprises, management of these multiple stressors needs to be based on a holistic approached founded on strong empirical evidence.

1.1 Types of stressor interactions

The generally accepted classification of interaction effects are synergistic (when the combined stressors effect is more than would be expected by the sum of individual effects), or antagonistic (when the sum of combined effects is less than would be expected for individual effects) (Crain *et al.* 2008; Darling & Côté 2008). Some authors have argued that this current classification is not adequate and that more categories of interactions for detecting them need to

be developed (Kath, Le Brocque & Maron 2014; Piggott, Townsend & Matthaei 2015). Having a robust conceptual and practical classification system of stressor interactions is imperative for effective management as it allows managers to identify stressors that could yield the most "positive synergistic" effects (Piggott *et al.* 2015). The authors identified a new class of "mititgating" synergisms that was previously classified as "antagonistic" following conventional classifications of multiple stressors and suggested that this type of synergism, which occurs when cumulative effects of stressors are reversed and amplified beyond the sum of individual effects in absolute terms, could have important management implications.

1.2 Pressing research needs to facilitate ecosystem management

Many authors have identified critical gaps in scientific knowledge that need to be resolved in order to facilitate assessment and management of cumulative stressors (Munns Jr. 2006; Halpern & Fujita 2013; Clarke Murray, Mach & Martone, Rebecca 2014). The most pressing are:

- Improving stressor and driver data availability;
- Improving habitat data availability;
- Linking activity drivers to pressures;
- Linking pressures to effects on ecological components;
- Increasing empirical knowledge of non-additive stressor interactions;
- Identifying non-linear responses;
- Understanding ecological response at various ecosystem scales (e.g. cellular, individual, population, community or ecosystem).

The gaps in knowledge with regards to stressor interactions have large management implications; the management of additive stressors is expected to be relatively straightforward, when stressors have synergistic or antagonistic interactions with other stressors, their management becomes more challenging, for example the removal of an antagonistic stressor could yield less than expected benefits while the removal of significant synergistic stressors could yield larger returns than expected.

Well-designed manipulative and natural experiments are fundamental tools to develop knowledge for the detection and quantification of stressor interactions (e.g. Silliman *et al.* 2005; Perkol-finkel & Airoldi 2010; Sundbäck, Alsterberg & Larson 2010; Falkenberg, Connell & Russell 2013). Data from multiple experiments can be analysed statistically using meta-analysis techniques to obtain effect sizes that can be used to predict interactions between specific

interacting stressors (e.g. Laskowski *et al.* 2010; Mantyka-pringle, Martin & Rhodes 2011; Harvey, Gwynn-Jones & Moore 2013). Moreover, empirical data from meta-analysis can form the basis for mathematical equations used in mechanistic modelling, or can contribute to modelling by providing initial values for states and determining constants (Osenberg *et al.* 1999). Mechanistic models can be used to predict responses to multiple stressors or management decisions (Weijerman *et al.* 2015). Meanwhile, the complexity of ecological responses and patchy data has prompted the development of formal methods and tools that allow researchers to tap into the growing pool of expert knowledge to enable the prediction of the likelihood of non-additive effects of multiple stressors (e.g. Catenacci *et al.* 2013; Ban, Graham & Connolly 2014).

Another important aspect of the multiple stressor discussion concerns the interactions between local and global stressors (Brown *et al.* 2013; Strain *et al.* 2015). The crux of the issue lies in the fact that local systems or habitats are impacted by multiple local (e.g. resource extraction, noise and chemical pollution etc.) as well as global stressors (e.g. climate change, ocean acidification etc.). Global stressors by definition lie outside the sphere of influence of local managers in terms of jurisdiction, and can have long management response timeframes due to the need for concerted global effort (Ghedini, Russell & Connell 2013). In the face of this challenge, one perspective in multiple stressor research highlights the potential role of managing local stressors that interact synergistically with global stressors in order to increase the resilience of local systems to withstand stress brought on by global changes (Brown *et al.* 2013). This "buys time" for local systems facing global stressors that require long timescales for resolution (Ghedini *et al.* 2013).

1.3 Mediterranean saltmarsh as model study systems

Human activities and urban development has focused mainly on coastal areas globally with large-scale loss and damage to natural coastal systems. Nowhere is this more true than the Mediterranean Sea which has been exploited since antiquity with serious consequences: fish stocks and predatory fish abundance have declined (Ferretti *et al.* 2008), food webs have been altered (Sala 2004; Lotze, Coll & Dunne 2011), coastal ecosystems are polluted and degraded (Airoldi & Beck 2007) and invasive species and diseases are rampant (Galil 2007). Important habitats such as seagrasses, saltmarshes and coastal lagoons have been reduced to <50% of their size at the beginning of the century, with losses >80 % in countries such as Italy (Airoldi & Beck 2007). Current knowledge suggests that the underlying drivers of change are a synergy of anthropogenic and natural stressors, including land reclamation, coastal development, overfishing, pollution, nutrient and sediment enrichment, and biological disturbances (Airoldi &

Beck 2007; Coll *et al.* 2012; Micheli *et al.* 2013). Saltmarshes are one of such natural systems that have undergone widespread replacement and face continued impact from increasing human pressures. Future climate alterations will impose additional threats to the persistence and stability of these habitats (Torresan *et al.* 2012; Bouma *et al.* 2014). Strong conservation and management actions are generally absent, and most of the recent degradation is attributable to failures of governance and implementation. An ad-hoc approach to managing multiple stressors is another obstacle to holistic management.

In the Adriatic Sea, saltmarsh systems are areas of high ecological importance that have traditionally undergone massive range restrictions and continued to be degraded by a myriad of stressors (Cencini 1998; Sarretta *et al.* 2010; Silvestri, Ghinoi & Barone 2013; Bellafiore *et al.* 2014), making them an ideal model study system. Saltmarsh vegetation are the ecosystem engineers that create saltmarsh habitats that are biodiversity hotspots and provide irreplaceable ecosystem services nutrient cycling, habitat provision and protection on storm surges and coastal erosion (Shepard, Crain & Beck 2011). Although most of the remaining saltmarsh areas in the Adriatic are now Natura 2000 sites as well as UNESCO World Heritage and Ramsar sites, they continue to face multiple human pressures.

1.4 *Spartina maritima* model study species

Among saltmarsh vegetation, grasses of the *Spartina* genus play an especially crucial role in coastal protection and habitat creation due to their extensive root and rhizome system which binds substrate together and perennial life history that enables the maintenance of the dense belowground root complex (Neumeier & Ciavola 2004; Widdows, Pope & Brinsley 2008). It is such an effective coastal stabiliser that the natural hybrid *Spartina anglica* has been purposefully transplanted in many coastal systems, sometimes with unintended negative ecosystem consequences (Thompson 1991; Hacker *et al.* 2001).

The *Spartina maritima* (Curtis) Fernald was first described by Fernald (1916). It is known commonly as the small cordgrass and is native to European coasts. It grows in tussocks of rigid shoots with strong roots and wiry rhizomes, with the short rhizomes forming secondary clusters close to the parent tussock. It is a perennial grass with heights ranging from 15 to 80 cm tall, with thin leaves ranging from 10 to 40 cm long with a tapered end. It produces flowers and viable seeds but spreads predominantly by vegetative clones (Marchant & Goodman 1969). New green culms grow in spring and summer and sequester carbohydrates in underground storage organs. The culms senesce in autumn and winter, leaving brown dried culms that are usually removed by the following winter storms. *S. maritima* can grow in single-species tussocks or in

mixed assemblage meadows with *Limonium*, *Halimione* and *Aster* in the *Puccinellia maritima* zone and around creeks and pools (Marchant & Goodman 1969).

The distribution of *S. maritima* in Europe ranges from the northern Spain and France to the Wadden Sea coast and southern England and Ireland; in the Mediterranean populations of *S. maritima* are found in the Adriatic Sea in Italy and Slovenia (Marchant & Goodman 1969). *S. maritima* has been in regression as a result of sea level rise and subsequent replacement by hybrids *S. townsendii* and *S. anglica* in the UK and displacement by *S. densiflora* in northern Spain (Castillo *et al.* 2000; Nehring & Hesse 2008). Genetic evidence gathered during the course of this PhD show the first evidence that *S. townsendii* and *S. anglica* have also established in some of the Adriatic saltmarsh systems (see Chapter 3- Article 5), although further work is needed to understand the exact distribution and abundance of these cryptic invasives, as well as to unravel the competitive interactions and differences in ecological function (if any) between *S. maritima* and its hybrids. Due to the high phenotypic plasticity of *Spartina* and the overlaps in physical characteristics and habitat occurrence between related hybrids, a positive species ID requires molecular genetic tests (Goodman *et al.* 1969; Baumel *et al.* 2003). As non-native *Spartina* was not known to occur in the study sites prior to the genetic study carried out in the third year of the PhD, work from the previous years (see Chapter 3- Article 3 which was published before the final results of the genetic tests) refer only to *S. maritima*, while subsequent chapters/ papers will refer to *Spartina* or *Spartina* spp.

In the Adriatic, *S. maritima* was also observed to be in decline somewhere around 2003, being replaced by the shallow-rooted perennial *Salicornia veneta* (Cazzin *et al.* 2009). As *S. maritima* tend to grow on the seaward edges of saltmarshes, they are especially vulnerable increasing erosion pressures as a result of vessel wake, increased storm frequency and intensity due to climate change, and relative sea level rise (as a result of a combination of global warming, land subsidence and low sediment supply). The provision of important ecosystem services like coastal protection (Neumeier & Ciavola 2004), nutrient absorption (Sousa *et al.* 2008) and carbon sequestration (Curado *et al.* 2013) combined with on-going threats from multiple stressors make *S. maritima* an important and relevant study species.

Figure 1 *Spartina* can be found growing at the lowest saltmarsh elevation, often along the seaward edge, forming single-species tussocks or meadows.

Figure 2 *Spartina* tussocks at the start of the growing season (June), seen here with macroalgal wrack accumulation.

Chapter 2 Thesis objectives, layout and approach

2.1 Objectives

1. Identify techniques that are used for the detection and prediction of cumulative stressor interactions
2. Develop new empirical knowledge through field experiments that contribute to increasing the understanding of multiple stressors in saltmarsh ecosystem
3. Analyse the rapid displacement of native *Spartina maritima* by cryptic, non-indigenous *S. anglica* in north Adriatic saltmarshes
4. Assess the spatial distribution of multiple stressors affecting saltmarsh vegetation and infer potential synergies from systematic review of multiple stressor studies in saltmarshes

2.2 Thesis layout, key research questions and approach

This thesis is structured around five chapters, with each chapter written as a stand-alone paper for publication. The following Figure 1 shows the structure of the thesis indicating the key research questions that guided the development of each individual chapter and main research methods used.

Article 1: Qualitative and quantitative methods for evaluation of interactions between stressors

The growing awareness of cumulative stressor interactions has led to a growing call for science to provide the tools for the adequate assessment of cumulative stressors and their potential interactions. Cumulative stressor analysis is currently a fairly new field with few standard methods of analysis, although new methods are being developed actively. I undertook this review to identify techniques for the detection and quantification of stressors interactions, as well as tools that allow the prediction of potential interactions.

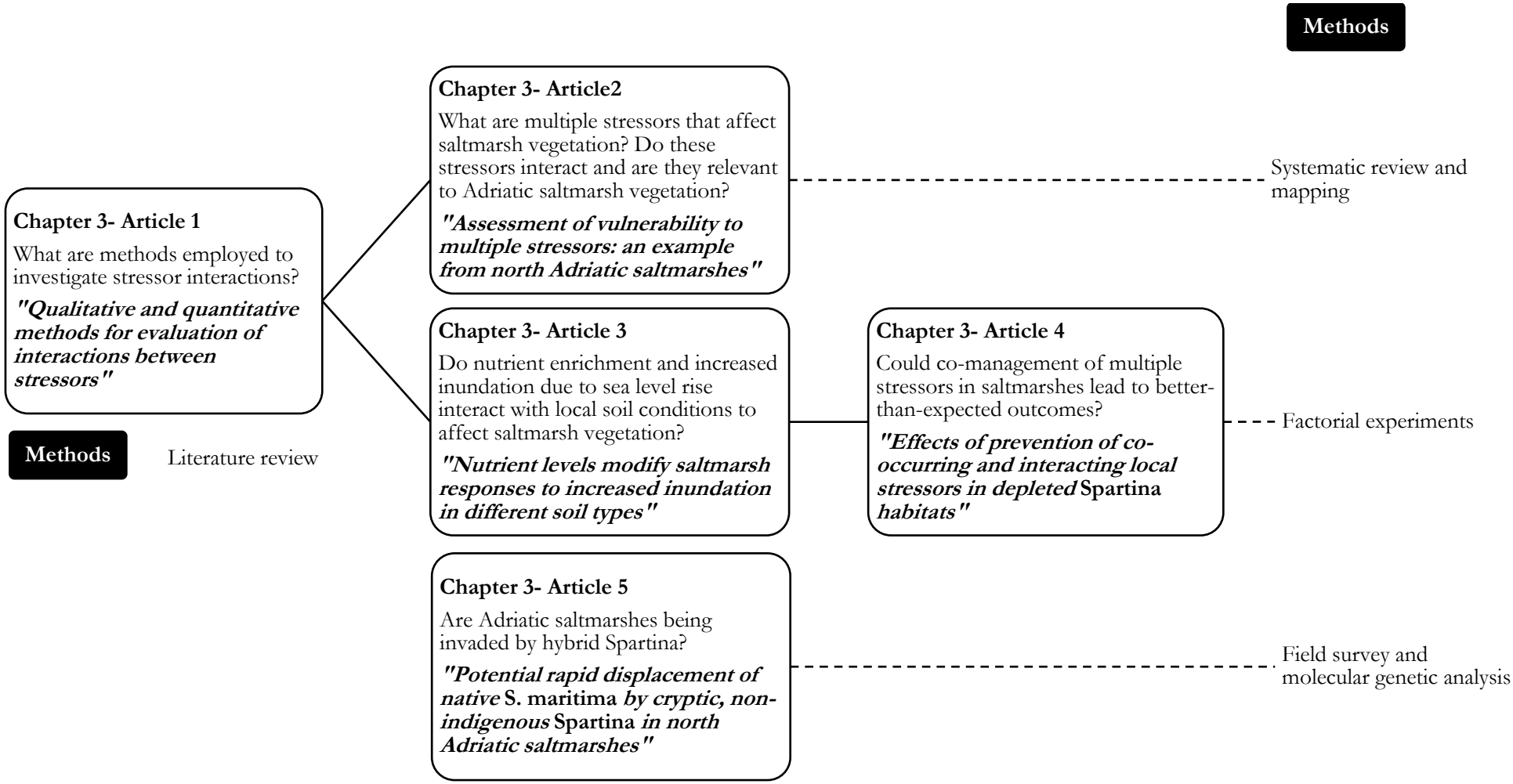

Figure 1 Structure of the thesis with key research questions, resulting article title and methods used to answer each research question.

Article 2: Assessment of vulnerability to multiple stressors: an example from north Adriatic saltmarshes

In this chapter I attempt to synthesise known information of saltmarsh pressures and drivers in the Adriatic and infer potential for important synergistic interactions based on saltmarsh multiple stressor experiments conducted globally. I used a methodology that combines both spatial analysis of pertinent saltmarsh vegetation stressors and systematic review to understand possible non-linear responses of co-occurring stressors.

Article 3: Nutrient levels modify saltmarsh responses to increased inundation in different soil types

Excess nutrients and increased inundation as a result of relative sea level rise are ubiquitous stressors in Adriatic saltmarshes and elsewhere. I conducted a field mesocosm experiment in Vallona Lagoon to assess the joint effect of increased nutrients and inundation on *Spartina* growth. Additionally, I tested whether responses were modified by soil organic content and plant origin. During this experiment, I unexpectedly found that *Spartina* sourced from different lagoons had markedly different survival, leading me to hypothesis that there could be genotypically distinct non-native *Spartina* in the study lagoons. This led to work in Chapter 3-Article 4 where I conducted a large-scale survey to test this hypothesis.

Article 4: Effects of prevention of co-occurring and interacting local stressors in depleted *Spartina* habitats

The bulk of stressor interaction knowledge available is based on experimental data from testing the joint effect of multiple stressors; the effect of management of multiple stressors is less tested. This is a key knowledge gap considering the pathways to recovery from disturbance can be different from the disturbance pathway. To identify potential beneficial effects of management of co-occurring stressors, I conducted a two-year field experiment to assess the joint effect of removing nuisance macroalgal wrack deposition and human trampling. These are two stressors co-occur during summer in the *Spartina* habitat in Bellocchio Lagoon, and being local in scale, are particularly relevant to local management considerations.

Article 5: Potential rapid displacement of native *S. maritima* by cryptic, non-indigenous *Spartina* in north Adriatic saltmarshes

Based on observations of differential survival of *Spartina* plants from different lagoons in a manipulative experiment (see Chapter 2), as well some preliminary genetic tests, I hypothesised that there could be more than one species of low intertidal *Spartina* in our study sites. Based on well-documented examples in the UK and the Wadden Sea, invasive *Spartina* could replace native *S. maritima* leading to substantial ecological effects. I conducted a large-scale survey of *Spartina* colonies in the main saltmarsh areas in the Adriatic in order to identify (using genetic methods) and qualitatively describe the occurrence of non-native *Spartina*.

Chapter 3 Articles

The following article (Article 3) was accepted for publication on 24 December 2014:

- Joanne X.W. Wong, Carl Van Colen, Laura Airoldi (2015) Nutrient levels modify saltmarsh responses to increased inundation in different soil types. *Marine Environmental Research*, 104, p. 37 – 46.

The following articles (Article 1, 2, 4 and 5) are in preparation:

- Joanne X.W. Wong and Laura Airoldi. Qualitative and quantitative methods for evaluation of interactions between stressors.

- Joanne X.W. Wong, Beth Strain, Laura Airoldi. Effects of removal of co-occurring and interacting local stressors in depleted *Spartina* habitat.

- Joanne X.W. Wong , Federica Costantini, Lorenzo Savelli, Danny Geelen, and Laura Airoldi. Potential rapid displacement of native *S. maritima* by cryptic, non-indigenous *Spartina* in north Adriatic saltmarshes.

- Joanne X.W. Wong, Mike Beck, and Laura Airoldi. Assessment of vulnerability to multiple stressors: an example from north Adriatic saltmarshes

Article 1 Qualitative and quantitative methods for evaluation of interactions between stressors

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Abstract

Increasingly, examples from laboratory and field experiments point to the presence of interactions between multiple stressors that can lead to unexpected ecological effects. These interactions have management consequences as synergistic stressors may lead to worse-than-expected effects while antagonisms may lead to deterioration if a mitigating stressor is removed. This has put a focus on the need for cumulative stressor analyses to detect and quantify interactions and increasingly, to predict the presence potential interactions before they can be detected. However, cumulative stressor analysis is a fairly new field with few standard methods of analysis, although new methods are being developed actively. This review was undertaken to identify case studies in which stressor interactions have been assessed either to detect/ quantify an interaction or to predict potential presence interactions. The diverse case studies include tried-and-tested techniques like experimentation and empirical as well as new methods like mechanistic modelling and Bayesian networks analysis.

A1.1 Introduction

Marine ecosystems globally are increasingly under threat from multiple human-driven stressors (Halpern *et al.* 2008). Effective conservation and sustainable use of marine environments will require a holistic ecosystem-based approach that considers all human uses and pressures on the system within the context of an interlinked ecosystem. Integral to this approach

is the management of cumulative stressors in an integrated manner instead of managing each marine use sector independently of other sectors (Kittinger, Crowder & Levin 2013).

Science has played an important role in creating tools and methods to identify, quantify and predict the effect of stressors through observations and experimentation. However, the prediction of the effect of even a single stressor is inherently complex as (1) response to a stressor is usually non-linear, such that dose-response cannot be accurately extrapolated from a subset of exposures (e.g. Ries, Cohen & McCorkle 2010), (2) receptors may exhibit temporal-dependent responses to a stressor (Thrush & Dayton 2002), (3) a stressor can seem to exert different effects on the receptor depending on the chosen response measure (i.e. physiological process rates, biomass or survival etc.) and level of organisation (i.e. effect at the cellular, organism or population level etc.) (Maltby 1999), and (4) a stressor may exert an effect on a receptor via indirect or higher-level pathways (e.g. altered predator-prey relationships) (Wootton 2007). The prediction of stressor effects is further complicated when multiple stressors co-occur (which is almost always the case in natural systems) as additional stressors can modify the effect of other stressors, such that the combined effect of stressors can sometimes not be deduced from summing single stressor effects (Folt *et al.* 1999; Crain, Kroeker & Halpern 2008; Darling & Côté 2008). Non-additive effects arise when a stressor (1) modifies a co-occurring stressor, for example polycyclic aromatic hydrocarbon contaminants in organisms can be excited by UV radiation leading to increased damage from excited molecules (Hooper *et al.* 2013), or (2) modifies the susceptibility or exposure rate of a receptor to another stressor, for example higher temperature increases the metabolic rate in mussels, leading to increase copper toxicity as a result of the higher exposure rate (Prasada Rao & Khan 2000).

Stressor interactions are commonly categorised as either additive, synergistic or antagonistic (see Figure 1). Stressors are "synergistic" when the sum of the effects is increased with reference to the effect of individual stressors and "antagonistic" when the sum of effects is reduced. In a meta-analysis study of multiple stressor experiments, Crain *et al.* (2008) reported antagonisms in 38% of studies and synergisms in 36% studies. However, Piggott, Townsend & Matthaei (2015) suggested that the current definition and calculation of interactions may be underrepresenting positive synergisms and over-representing negative synergisms. The authors showed that there are many more subsets of possible interaction types, pointing out specifically the case of "mitigating synergies" where two stressors that individually have effects in the same direction when combined can have an opposite effect; they suggested that more robust definition and systematic classification of interactions is an essential step in predicting and managing interactions.

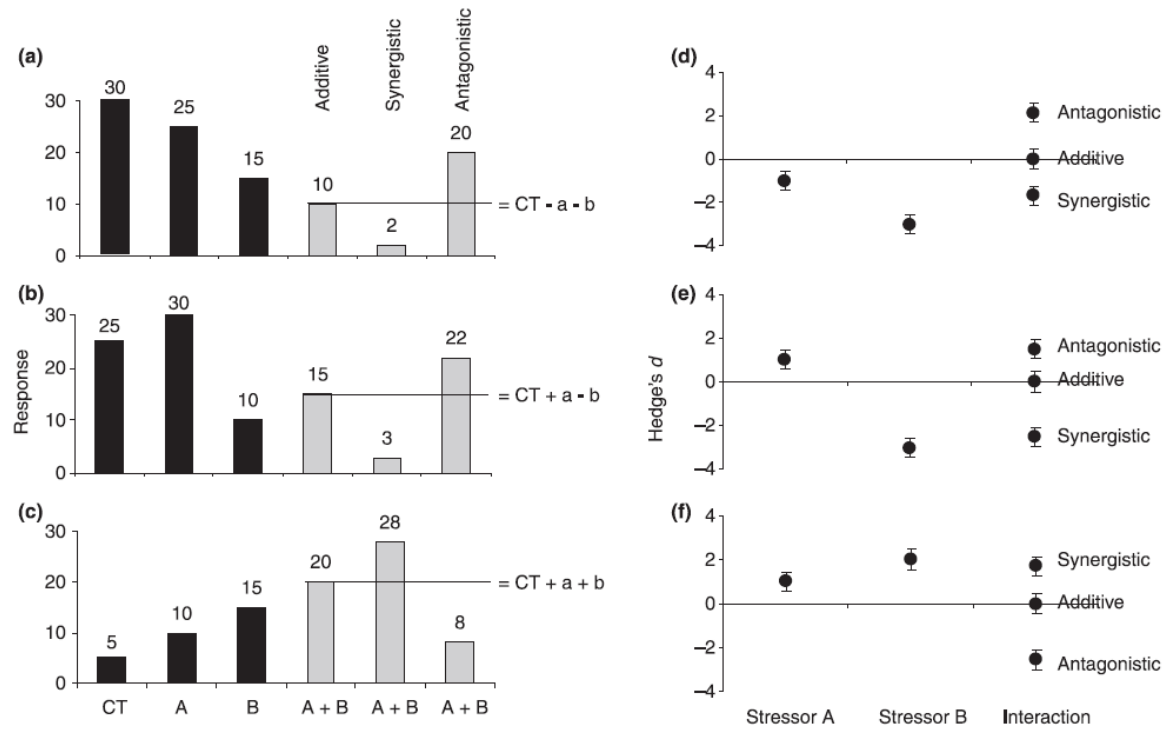

Figure 1 Conceptual representation of treatments in factorial studies with four treatments: control (CT), plus stressor A (A), plus stressor B (B), and with both stressors (A + B) (a, b,c). The A + B response is used to catagorise the interaction type (additive, synergistic or antagonistic) (d,e,f). Example represent stressors that have double negative (a and d), opposing (b and e), and double positive (c and f) main effects on a response variable (from Crain *et al.* 2008).

There is an increasing awareness of the presence of complex stressor interactions and the potential for these interactions to cause unexpected 'enigmatic' ecological effects (Raiter *et al.* 2014). This has led to a push in legislation requiring adequate analyses of cumulative stressors and potential interactions within project-level impact assessments well as region-wide ecosystem assessments to support ecosystem-based management and decision-making (e.g. Canada's Eastern Scotian Shelf Integrated Ocean Management Plan (Fisheries and Oceans Canada 2007) and Australia's Great Barrier Reef Marine Park Cumulative Impact and Structured Decision-Making framework (Anthony *et al.* 2013)). While legislation in many countries now require the assessment of cumulative stressor effects and interactions in project-level impact assessments, in practice, these are often not assessed adequately (Morgan 2012), in large part due to large knowledge gaps of interaction processes as well as a mismatch in assessment scale since project planning and assessment operates at a smaller-scale compared to the larger scale in which

multiple stressor effects can manifest. Meanwhile, cumulative stressor analysis in regional ecosystem assessments seems to be advancing with much more headway (Duinker *et al.* 2012).

Although a much guidance material have been developed to guide cumulative effect assessments at the project and regional scale, these tend to be over-arching frameworks or approaches (e.g. Kingsley *et al.* 1999; Connelly 2011; Cardinale & Greig 2013; Natural England 2014) that provide little concrete advice on how exactly to identify and predict cumulative stressors and there interactions (with a few notable exceptions (see Anthony *et al.* 2013; International Council for the Explorations of the Sea (ICES) 2014)). Indeed, some reviewers have commented that scientific knowledge is not keeping pace with laws, leading to insufficient tools and guidelines particularly for quantifying cumulative effects and interactions (Munns Jr. & Jr 2006; Kappel *et al.* 2009). We undertook this review in order to provide an overview of methods that have been employed to detect, quantify and predict cumulative stressor effects. For this review, we define a stressor as either an abiotic or biotic variable that exceed its normal range of variation, with negative effects on individual physiology, population performance or ecosystem functioning in a significant way (Vinebrooke *et al.* 2004).

A1.2 Approaches in detecting, quantifying and predicting stressor interactions

There are generally two broad goals in stressor interaction studies (Figure 2). The first goal is to detect and quantify stressor interactions while the second is to predict the likelihood and type interactions to occur. Appropriate methods will depend on the goals of the analyses as well as the type of data being considered. Manipulative and natural experiments combined with statistical analysis are still the most important ways in which specific stressor combinations can be tested to detect and quantify non-additive effects. Results from multiple experiments can also be combined across studies using meta-analysis to derive the overall effect size of interaction. This method in effect increases the study sample size of stressor pairs so that general conclusions may be drawn about interaction types between stressor pairs. There are currently limited techniques that allow the detection of stressor interactions based on non-experimental data. One such method is the use of regression trees to model correlations between an observed response with measurements of multiple factors (e.g. physical stressors); this method also allows for the detection of interactions between factors (De′ath & Fabricius 2000). However, this method only shows correlation not causation, significant interactions would still need to be experimentally

tested and confirmed (*ibid*). Empirical, mechanical and hybrid models have been used both for predicting the presence of potential interactions between stressors, as well as the responses to interactive effects of multiple stressors. Bayesian networks analysis a type of probabilistic modelling that has been used to assess the likelihood of stressor interactions under different stressor combinations. In the following sections, we demonstrate the use of these various methods in stressor interaction studies illustrated with case study examples from recent studies from both published and grey literature.

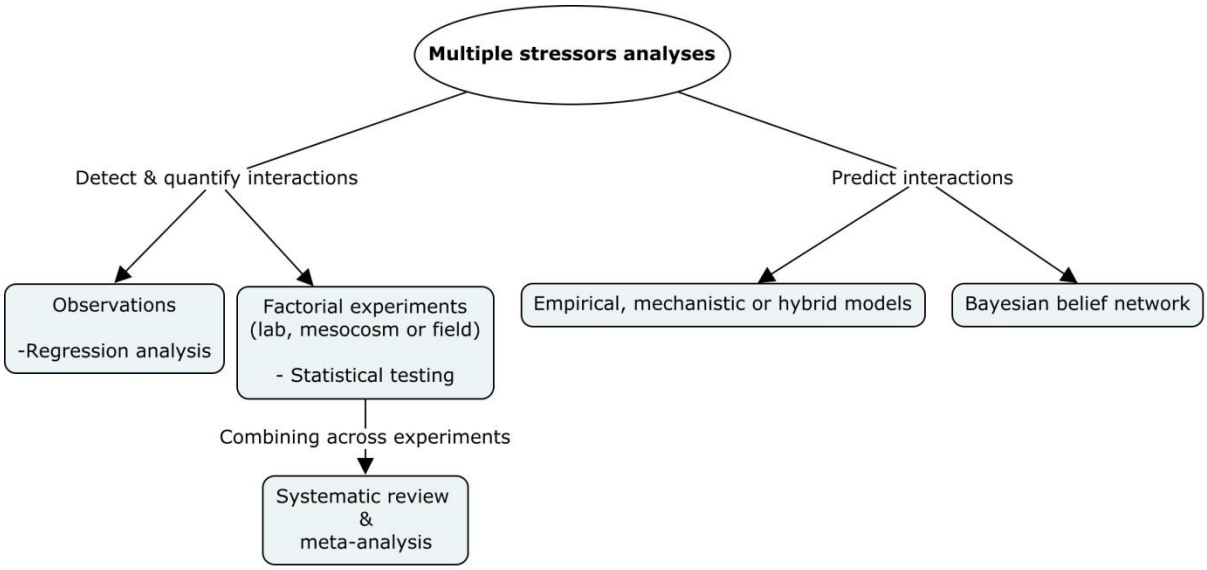

Figure 2 Main techniques and methods used to detect, quantify and predict multiple stressor interactions.

A1.2.1 Planned and natural experiments

Experiments with multiple stressors have greatly improved our knowledge of how stressors modify the effect of other stressors. The most basic experimental design for the detection of interaction between two factors is a randomized manipulative experiment where the two factors contain two levels each and all four possible combinations of treatments are tested. Manipulative experiments can be conducted in the laboratory (e.g. Russell *et al.* 2009) or in a field setting (e.g. Nelson & Zavaleta 2012). Well-designed natural experiments based on observations of natural variations of different factors can produce datasets that are structurally similar to randomized manipulative experimental results (e.g. Darling, McClanahan & Côté 2010).

The majority of factorial experiments until recently have been analysed using the classical statistical technique of analysis of variance (ANOVA). In the most simple design with two factors of two levels each, the ANOVA allows the test of three null hypothesis: (1) there is no difference between levels of factor A independent of factor B; (2) there is no difference between

levels of factor B independent of factor A, and (3) differences among levels of factor A (if they exist) are independent of differences among levels of factor B (if they exist). Rejecting the third null hypothesis would be equivalent to detecting a significant interaction between the two factors (Dunne 2010) (Figure 3). The use of ANOVA requires that a few assumptions be fulfiled, including normal distribution of sampled means and linear response to the factors over the ranges examined. When these assumptions are violated, generalized linear models may be the more appropriate statistical analysis (Dunne 2010).

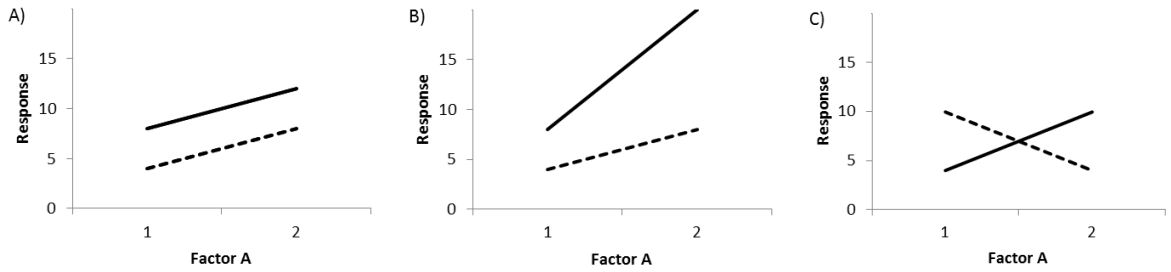

Figure 3 Interaction plots showing response to Factor A at level 1 (solid line) and level 2 (dotted line) of Factor B. In (a) there is no interaction and main effects of Factor A and B can be interpretted from the ANOVA. If an interaction between Factor A and B is significant, the type of interaction, i.e. (b) synergy or (c) antagonism, will need to be visualized using interaction plots (after Dunne 2010).

The main advantage of planned experiments is the ability to hold other factors constant so that one can manipulate only the factor of interest and examine the response of the subject to levels of the factor of interest. Laboratory-based microcosms and mesocosms also allow researchers to manipulate factors that would be impossible or very difficult to manipulate in a field setting, for example CO_2 and temperature increases (Benton *et al.* 2007). However, this same point could also be a disadvantage as laboratory-based experiments do not reflect reality where organisms exist within an ecosystem and is subject to a large number of factors and interactions with other organisms. Particularly for situations where there are many higher-order interactions in the natural field setting, results from mesocosms may not reflect reality (Skelly 2002). A well-designed experiment that includes both laboratory-based experiments and field-based mesocosms can have the best of both methods while simultaneously overcoming the individual disadvantages (Odum 1984).

A1.2.2 Meta-analysis of multiple experiments

Ecological experiments have generated a large repository of data in the form of published papers. These papers were traditionally reviewed and discussed qualitatively, however, results between studies are seldom directly comparable due to different magnitudes of effects and experimental designs. Further, qualitative comparisons of results would become quickly intractable with increasing numbers of studies. Since the late 1990s, meta-analysis has been used to analyse combined experimental results in an objective, systematic and transparent way. Meta-analysis is a set of statistical methods that is used to combine data across studies in order to obtain general conclusions from many separate studies (Arnqvist & Wooster 1995).

Meta-analysis has grown in traction in ecology because it has been valuable for increasing power, exploring heterogeneity, detect higher-order trends and is consistent with and evidence-based decision-making approach (Stewart 2010). Multiple stressor meta-analyses often employ the method by Gurevitch, Morrison & Hedges (2000) that allows the calculation of the individual and main effect sizes of two factors as well as the interaction effect size if the experiment is of a 2 x 2 design (i.e. four treatments: control, plus stressor A, plus stressor B, and with both stressors) (e.g. Crain *et al.* 2008; Darling *et al.* 2010; Harvey, Gwynn-Jones & Moore 2013; Rosenblatt & Schmitz 2014).

A1.2.3 Regression trees

Regression trees have been used to explore interactions between variables that explain a response. It can be used to analyse a set of observational data and does not require datasets from orthogonal multiple stressor experiments. Thus, it can be used to detect interactions between variables in long-term monitoring datasets. Trees explain variation of the response variable by "repeatedly splitting the data into more homogeneous groups, using combinations of explanatory variables that may be categorical and/or numeric" (De'ath & Fabricius 2000). The resulting groups are described by a typical value of the response variable, the number of observations in the group, as well as the values of the explanatory variables that define it (De'ath & Fabricius 2000). Parravicini *et al.* (2011) used regression trees to analyse the relationship between habitat status score (response variable) and eight different human uses pressures (explanatory variables). It was possible to deduce from the results that pairs of human use pressures were mainly additive, except beach re-nourishment and the commercial harbour which were strongly synergistic in driving the habitat status of the studied coastal area.

Regression trees are effective alternatives to other commonly used methods like regression models. They are also able to highlight complex interactions and to handle various data types and handle missing values (De'ath & Fabricius 2000; Parravicini *et al.* 2011).

A1.2.4 Model for predicting presence interaction effects

Models are generally divided into two groups: empirical (or statistical) and mechanistic (or process) models. However, in reality most models are hybrids of both empirical and mechanistic models (Duarte *et al.* 2003). Empirical models are based on the statistical relationships between different variables. An example would be a model that regression model that statistically represents the relationship between chlorophyll *a* concentrations and dissolved phosphorus. Such a model may accurately predict the response in chlorophyll *a* for different levels of dissolved phosphorus if the level of dissolved phosphorus was included in the range used to formulate of the model. However, since it does not "explain" the underlying mechanisms that cause the change in the response, the model could fail if applied to predict the response to conditions outside the "environmental envelope" used to estimate the model parameters (Duarte *et al.* 2003). While empirical modelling are useful for describing existing trends in the data, confidence in outcomes decreases as the system moves further away from the original conditions used to construct empirical relationships (Sutherland 2006; Menzie, MacDonell & Mumtaz 2007; Woodin *et al.* 2013). Techniques for empirical modelling include multivariate regression and principal component analysis (Menzie *et al.* 2007). Mechanistic models on the other hand relate variables in the dataset with functions specified by biological processes that are believed to have given rise to the data. As such, all parameters in the model have biological definitions. Mechanistic modelling is seen by some as having more robust predictive capabilities than empirical modelling; since mechanistic models have a basis in underlying biophysicochemial processes they can retain some level of mechanism constancy when faced with new objects or conditions (Matzelle *et al.*; Keller 1989; Menzie *et al.* 2007; Leites, Robinson & Crookston 2009; Todgham & Stillman 2013). Accurate mechanistic models are difficult to create due to large data requirements such that only the most well-studied species or stressors can be modelled (Pearson 2008). Mechanistic models can also fail when underpinning processes that are used to define model parameters change under new environmental conditions (Woodin *et al.* 2013).

Complex hybrid and mechanistic models have been used to predict the potential presence of interactions between stressors. This is based on the premise that accurate models are good representations of reality and would be able to "react" in a realistic way to simulated perturbations. In this way, combinations of perturbations can be simulated and potential interactions between perturbations would manifest in simulated responses. One example used a coupled biogeochemical and food web model to investigate potential joint effects of fishing management (50%, 30% or 20%) and nutrient management (business-as-usual or reduction) on

cod biomass. Stressor interactions were indicated by comparing the model output for different scenarios (Niiranen *et al.* 2013; International Council for the Explorations of the Sea (ICES) 2014). Another example used an ecosystem model to deduce potential interactions between fishing, ocean warming and ocean acidification on multiple functional groups by comparing model results with different stressor combinations. Interaction size and direction was calculated for the ecosystem and communities by comparing responses to stressors in combination and singly and in pairs (Griffith & Fulton 2014). Similarly, Cornwall & Eddy (2014) investigated the cumulative effect of ocean acidification and removal of fishing pressure using an Ecopath and Ecosim food web model. Stressor interactions on biomass and trophic level were calculated based on the difference between the model-predicted effects of Stressor 1 + Stressor 2 compared to the additive effect of single stressors.

A1.2.5 Bayesian model for predicting likelihood of interactive effects

Bayesian network (BN) analysis (a.k.a. Bayesian Belief Networks), are "probabilistic graphical models that represent a set of random variables and their conditional interdependencies" (Pollino & Henderson 2010). The BN is made up of nodes that represent factors or outcomes of interest (e.g. salinity, a measure of health, occurrence of an event etc.) and links between the nodes which represent informational or causal dependencies between the nodes (Pearl 1997). BNs have found increasing application in environmental sciences in the last decade, e.g. for making predictions and characterisation of systems, due to increased computational power (Aguilera *et al.* 2011).

BN analysis in multiple stressor studies usually starts with the creation of a conceptual model of the system with the key drivers, pressures and state changes as nodes, and the interlinkages between the nodes. DPSIR tables can also be set up as nodes and linkages in BNs (Barton *et al.* 2012); nodes in BNs can represent drivers, activities, pressures and ecosystem values, while linkages between nodes represent impacts and process (Anthony *et al.* 2013). The conceptual model is then refined into an influence diagram where probabilities are then used to characterise the strength of relationships between nodes (Pollino & Henderson 2010). Nodes and probabilities can be populated using observed data or data extracted from literature reviews (e.g. Anthony *et al.* 2013; Kath *et al.* 2014). Data elicited from experts can also be incorporated in BNs and can be used to overcome problems of scarce data, or when data is complex and equivocal (International Council for the Explorations of the Sea 2014). This is particularly well-suited to the conceptual model construction stage (e.g. Ban *et al.* (2014b); International Council for the Explorations of the Sea (2014) Model 2). However some drawbacks of using expert

opinion are that expert opinion can be subjective and contradictory, and may not be representative of reality (Sutherland 2006).

BNs can be used to predict the probability distribution of how likely different effects are to occur at different combinations of two stressors. By comparing changes in likelihood of outcomes with different stressor combinations, Kath *et al.* (2014) used BNs to estimate potential interaction effects of stressors in combination. BN analysis can also be used to "detect" potential interactions that are believed to have the potential to occur. This method was employed by Ban *et al.* (2014b) to "detect" stressor interactions based on beliefs of experts. A summary of BN analysis to investigate stressor interactions can be found in Table 1.

Compared to mechanistic models, BNs are time- and cost-effective and have the ability to represent uncertainty and variability explicitly (Mccann, Marcot & Ellis 2006). It is highly flexible in allowing the use of diverse types of information (theoretical, mechanistic and empirical) (Clark & Gelfand 2006). It is also able to handle situations where some variables/ links are data rich while some are data-poor (Ban *et al.* 2014).When influence models and probabilities are developed jointly by different stakeholders, it provides an opportunity for social learning (Barton *et al.* 2012). BNs also allow the incorporation of prior knowledge in model development and new knowledge into existing models, and formal procedures to illicit expert judgement is being actively developed (Berger 2000).

Table 1 Case studies of Bayesian network analyses

Reference	Aim	How was stressor interaction assessed?	Approach	Variables of interest	Ecosystem type	Assessment timeframe	Spatial scale
Model 2 in ICES (2014)	Assess the effect of combinations of fishing management (Business As Usual, 30% cut or 60% cut) and nutrient management (Business As Usual, 30% cut or 60% cut).	Stressor interaction was implicit in experts' assessment of likelihood and direction of environmental change at various combinations of levels of stressors.	Fourteen experts were surveyed to populate a BN	Stressors: Fishing and nutrient enrichment Response: Overall ecosystem status (described using 5 categorical qualitative classes).	Benthic-pelagic component if Baltic Sea food chain	Assessment point was 20 years after management actions are fully implemented	The Baltic Sea, onshore and offshore
Ban *et al.* (2014b)	Obtain estimates of outcomes for different combinations of levels of stressors and their management options.	Experts were asked to assess outcome likelihoods for different combinations of stressors and management actions. Perceived likely interactions can be deduced from difference in response when both stressors are increased relative to just one.	BNs was created using empirical data for nodes for which data was available (i.e. physical and climatological nodes), and expert opinion when empirical data was not available. Experts were asked to rate the likelihood of outcomes on four responses under different combinations of stressors.	Stressors: Various anthropogenic stressors, environmental stressors and water quality Response: Probabilities of bleaching, disease outbreak, crown-of-thorns outbreak and hard coral cover decline	Mid-shelf coral reefs	10 years	Great Barrier Reef
Kath *et al.* (2014)	Demonstrate the use of BNs to identify stressor interactions	The response and type of interaction was identified by comparing the response of *E. camaldulensis* to changes in a single environmental factor under different states of a second environmental factor.	BN was used to relate *E. camaldulensis* abundance and condition in 37 wetlands to 10 environmental factors. The environmental variable data were extracted from existing spatial data, satellite imagery as well as measured data.	Ten environmental factors: lateral connectivity, groundwater depth past (1987), groundwater depth recent (2009), long-term rainfall, distance from weir, inundation frequency, agricultural land cover, remnant vegetation cover, grazing intensity and canopy cover Response: *Eucalyptus camaldulensis* Dehnh. abundance and condition	Floodplain wetland	Not specified	Thirty seven wetlands from the Condamine catchment (24434 km^2).

A1.3 Discussion

There is a strong consensus across ecological sciences that empirical testing under controlled laboratory, mesocosm and field settings are essential for understanding the impact of multiple stressors (EFSA 2013; Jutterström *et al.* 2014), particularly since we are not yet able to predict cumulative stressors from single-stressor effects (Todgham & Stillman 2013). However, due to the complexity of natural systems and the plethora of human and natural factors that impose stresses on the systems, it is technically impossible to experimentally test all types and levels of stressors on ecosystem receptors. New methods of using highly-calibrated ecosystem models to investigate stressor interactions (as well as management action interactions) could be a way forward. Potential interactions, as identified by the model, are useful in developing hypothesis for further testing.

The various techniques used in cumulative stressor analysis can be complementary and analyses usually involved a combination of methods. Meta-analysis was used to synthesise empirical data from experiments and long-term observations to provide direct inputs into mechanistic models and BNs. Canvassing experts for opinion, and to a lesser extent stakeholder consultation, remain integral parts of cumulative stressor analysis, particularly for the conceptual modelling of the system for BN analysis. The Baltic Sea pilot project (International Council for the Explorations of the Sea 2014) shows how this can be achieved in a transparent and systematic way.

BNs have been shown in recent applications to be potentially important tools in assessing stressor interactions. Using BNs, the International Council for the Explorations of the Sea (2014) and Ban *et al.* (2014b) evaluated stressor interactions via expert opinions of likelihood of outcomes under different combinations of stressors, while Kath *et al.* (2014) evaluated stressor interactions by comparing likelihoods of outcomes under single vs. paired stressor combinations based on observed data.

Cumulative stressor studies are an active field of development with new methods being developed and trialled to overcome the issue of imperfect scientific knowledge about stressor interactions. While more empirical research is clearly needed, more effort should go into making existing research results accessible and usable, which will facilitate the use of empirical data in multiple stressor analysis. At the same time, research should continue to development new methods that can utilise existing knowledge for cumulative effect assessment. Studies that use

mechanistic modelling and experimentation tended to assess smaller numbers of stressors, while regression trees and BNs were able to handle a larger number of stressor variables.

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Article 2 Assessment of vulnerability to multiple stressors: an example from north Adriatic saltmarshes

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Abstract

A key challenge of ecosystem management is to determine which stressors are affecting the system. We present a methodology for analyzing the vulnerability to multiple stressors that combines both spatial information on current impacts and an understanding of the possible non-linear responses to co-occurring stressors. In order to infer potential synergistic interactions between stressors, we first conducted a systematic review of multiple stressor experiments on saltmarsh vegetation. Next, to identify multiple stressors relevant to our study area we conducted a qualitative review of site-specific saltmarsh literature. Based on this list of locally-important stressors, we conducted a spatial analysis where we mapped the distribution of the stressors onto saltmarsh receptors based on publicly-available data. The resulting stressor maps are discussed with reference to potentially synergistic interactions identified in the systematic review. From the systematic review, the most-tested stressor was salinity (with most studies pairing salinity with inundation or temperature). However, salinity change has not been widely studied as a saltmarsh stressor in the study site, leading to little overlap between meta-analysis and locally-important stressors. The main pressures identified in the study-site were increased wave and wake energy, sediment loss from the lagoonal system, land subsidence and sea level rise. The spatial analysis revealed the lack of publicly-available pressure data; but available data indicate that there could be stressor hotspots pertaining to wind waves, nutrient

enrichment and vessel wake. Additionally, we discuss potentially important stressors that are currently little-researched in the study site.

A2.1 Introduction

As ecosystems globally face increasing human alteration (Vitousek et al. 1997), there is an urgent need to increase our understanding of the ecological consequences of these stressors in order to avoid unwanted ecological surprises (Paine, Tegner & Johnson 1998). Potential synergistic effects between multiple co-occurring stressors further underscore this urgency; non-additive interactions were present in approximately three quarters of studies examined by Crain et al. (2008) and Darling and Côté (2008), with synergies almost as common as antagonisms. In saltmarsh systems, unexpected synergies (where cumulative effects were greater than expected between stressors have led to large reduction in saltmarsh cover. In south-eastern USA, drought-induced stress and snail grazing (which increases fungal growth in wounds on leaves) interacted synergistically to induce massive die-offs of *Spartina alterniflora* cordgrass meadows (Silliman *et al.* 2005). Another study found interactions between stressors that occurred at different times; in Cape Cod, USA, mosquito control ditches dug in the 1930s led to the replacement of high marsh habitats by the low marsh *S. alterniflora*. Decades later, heavy fishing released the herbivorous crab *Sesarma reticulatum* from predator control. The proliferation of *S. reticulatum* which favours S. *alterniflora* resulted in large areas of denuded low marsh habitat (Coverdale *et al.* 2013). The understanding of cumulative stressor effects is needed not just to avoid ecological surprises, but forms an important part of conservation effort prioritisation (Diefenderfer *et al.* 2009; Allan *et al.* 2013). Depending on management goals, cumulative stressor analysis can be combined with ecological services assessment to identify low stressor/ high services sites for prioritisation, or identification of areas most vulnerable to multiple stressors that could benefit from management (Allan *et al.* 2012).

Saltmarsh vegetation provide important ecosystem services such as storm surge protection (Barbier *et al.* 2011; Möller *et al.* 2014), promotion of sedimentation (Mudd, D'Alpaos & Morris 2010), carbon accumulation (Nyman *et al.* 2006; Laffoley & Grimsditch 2009), stabilization of saltmarsh soil by roots (Francalanci *et al.* 2013), and functioning as nutrient sinks (Jordan, Stoffer & Nestlerode 2010). By modifying these physical processes and resource flows, saltmarsh vegetation plays an important role as ecosystem engineer that creates suitable conditions for many associated species and should be prioritised in conservation management (Crain & Bertness 2006). Unfortunately, saltmarsh habitats worldwide have been and continue to be under multiple anthropogenic stressors (Adam 2002; Gedan, Silliman & Bertness 2009),

threatening their continued ability to provide important ecosystem services.

We present a methodology for analysing the vulnerability to multiple stressors that combines both spatial information on current impacts and an understanding of the possible non-linear responses to co-occurring stressors in north Adriatic saltmarshes. Our approach comprised three main components. First, in order to be able to evaluate potential synergistic interactions between co-occurring stressors, a systematic review of empirical multi-stressor saltmarsh studies was conducted. Next, we attempted to assess multiple stressors in the Adriatic saltmarshes. To do this, we conducted a qualitative review of saltmarsh-relevant studies from the Marano Grado Lagoon, Venice Lagoon and the Po Delta. Then, using publicly-available data, stressors were mapped onto known saltmarsh receptors in the study area to identify multiple stressor hotspots and to assess qualitatively the potential for stressor interactions. The ecological importance and highly human dominated nature of these saltmarshes make them ideal study sites for this analysis.

A2.2 Methods

A2.2.1 Systematic review

We conducted a systematic review of saltmarsh multiple stressor experiments to obtain an overview of the state of knowledge regarding the effect of multiple stressors. Empirical experiments that manipulated two or more stressors affecting saltmarsh vegetation were identified using the Web of Knowledge database. Papers from 1950 to 2012 were included in the search. We narrowed down 13 separate stressor-types (see Box 1 for search keywords) that represent key drivers/ pressures that are currently threatening saltmarsh vegetation worldwide (e.g. Adam 2002; Gedan *et al.* 2009). We searched for articles with both saltmarsh habitat-related and stressor-related search terms under Topic (Box 1). We attempted to use words that describe saltmarsh pressures (e.g. trampling, nutrient, grazing etc.), however, this was not always feasible. For example, for the pressure of fish resource extraction, we used the search term "fishing" which is a driver instead of a pressure because we did not find an appropriate pressure search term. We also felt that papers that dealt with pressures arising from fisheries resource extraction would likely include the term "fishing" in the topic. We used the search-terms "erod*" and "eros*" (to describe erosion) which are terms related to state changes because we did not find suitable pressure-related search terms; using pressure search terms for increased wave, wind or vessel wake or increased currents would return too many studies to be tractable. Again the rationale was that papers dealing with pressures related to erosion would likely include this state change term.

To be considered relevant, experiments had to assess two or more stressors factorially and report the significance of statistical interaction between stressor pairs. A paper can have more than one experiment if more than two stressors were tested factorially. Experiments where the interaction between stressor pairs was not explicitly reported were not assessed. Only experiments that measured the response of saltmarsh vegetation were included in the systematic review since we were interested in the effect of stressors that reduced the capacity of saltmarsh vegetation to function as ecosystem engineers (i.e. creating physical habitat). Thus, studies related to saltmarsh fauna and algae only were not included. When multiple end-points were measured, the measures were prioritised in the following manner: biomass (total assemblage, species, individual plant, shoot, root), plant survival, germination rate and other plant measures (e.g. height, leaf area etc.). Each study could yield more than one test if multiple species were tested and reported separately, or if there is a third factor was tested and reported separately. Due to the low numbers of studies found, we did not conduct meta-analysis to determine the overall interaction effect size of stressor pairs. Instead, the presence or absence of significant interaction and the associated significance value was tallied for each experiment and the general trend for interaction was discussed qualitatively.

Box 1 Search terms used for the systematic review

The following habitat-related keywords were used in every search:
"saltmarsh" OR "salt marsh" OR "saltmarshes" OR "salt marshes" OR "oligohaline marsh" OR "oligohaline marshes"

We focused on the following 13 stressor types that are believed to have negative effects on saltmarsh vegetation. We searched for the following stressors in pairs in order to identify studies that included at least two stressors:

Stressor type	Search terms
Trampling	trampl*
Erosion	eros* OR erod*
Increased nutrients	nutrient* OR eutroph*
Wrack	wrack
Debris	debris
Grazing	graz* OR herbiv*
Harvesting of vegetation	mow* OR cut* OR harvest* OR hay*
Temperature	temperature
Salinity	salinity
Fishing	fishing*
Invasive pressure	introduced OR invasive OR "non-native" OR "non native"
Toxicants	toxic* OR contaminant* OR oil
Increased inundation	inundation OR "sea level rise" OR "sea level increase" OR "rising sea level" OR "increasing sea level"

A2.2.2 Multiple stressors in Adriatic saltmarshes

Study area

The saltmarshes in the Italian Adriatic form the main saltmarsh complex in Italy, and is one of few in the European Mediterranean (Dijkema *et al.* 1984). These saltmarshes, together with the Commacchio brackish wetland, are probably the second most important wintering site

for waders in the Mediterranean (Baccetti *et al.* 1996). The main saltmarsh systems considered in this study were (1) the Marano Grado Lagoon, (2) the Venice Lagoon and (3) the Delta Po saltmarshes (Figure 1). These saltmarshes support important economic activities including aquaculture, fisheries, tourism and transport (Fondazione Ca' Vendramin 2010; Silvestri, Ghinoi & Barone 2013). The saltmarsh cover that remains today is only 30% of that from the turn of the century, having been lost to land-conversion and hydrological management (Cencini 1998). All three saltmarsh systems have been highly modified by human intervention in the past and continue to be subject to a plethora of anthropogenic stressors like erosion from vessel wake (Silvestri *et al.* 2013), lack of sediment input to support vertical accretion (Fagherazzi *et al.* 2013), legacy heavy metal pollution (Emili *et al.* 2013) and accumulation of plastics and other pollutants (Vianello *et al.* 2013). Sediment deficit together with rising sea levels from climate change now threaten the very existence of these wetlands. In the Venice Lagoon, it is estimated that most of the saltmarsh habitats will not be able to maintain their elevation with regard to sea level given the Intergovernmental Panel on Climate Change (IPCC) "best estimate" rate of sea level rise (Day *et al.* 1999). Active recreation of artificial saltmarsh islands using dredge material have been carried out in Venice Lagoon and some Po Delta lagoons to stop or reverse its decline (Fondazione Ca' Vendramin 2010).

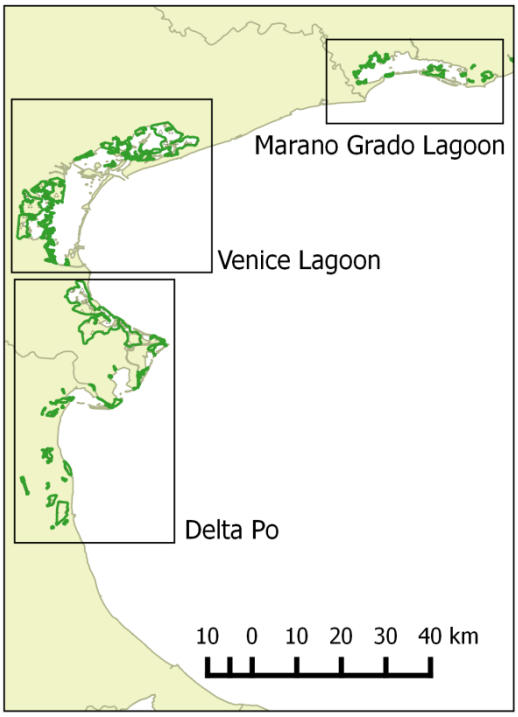

Figure 1 Study area: three main saltmarsh areas in the Italian Adriatic coast that was the focus of this analysis. Saltmarsh cover (in green) from Corinne land cover map (2006) (EEA).

Identifying site-specific drivers and pressures of concern

The first step in our approach was to identify the known drivers and pressures that are having negative effects on saltmarsh vegetation state in the Italian Adriatic. A qualitative review of published and grey literature was conducted using Google Scholar for papers or articles that identified stressors that were threatening the growth or persistence of saltmarsh vegetation in the study area. We used relatively broad search words like "stressor", "impact", "saltmarsh" together with "Adriatic" or "Italy" in order to capture as many articles as possible dealing with negative effects on saltmarshes in the study area. Articles were only included if there was a clear link between the stressor and a negative effect in saltmarsh vegetation in terms of reduction in abundance, growth or cover. Articles collected were reviewed to extract information to populate a conceptual model illustrating key linkages between the causes of stressors and their effects on saltmarsh state. Conceptual models are critical tools for multiple stressor assessments due to its ability to convey complicated interactions in a visually effective way (Menzie, MacDonell & Mumtaz 2007). The model was created using the CMAP tool (www. cmap.ihmc.us).

The components of the conceptual model were defined based on the DPSIR (Driver-Pressures- State Change- Impact- Response) framework (EEA 2007). The framework helps structure thinking about the interplay between human activities and environmental consequences and facilitates the simplification of complex linkages (EEA 2007; Niemeijer & De Groot 2008). As there are many variations of the DPSIR framework (Gari, Newton & Icely 2015), it is important that definitions of terms used are clear. In this regard, we follow the definitions laid out in EEA (2007). Drivers (D) are socio-economic sectors that fulfil human developmental needs, for example for food and shelter. Pressures (P) are caused by human activities arising from drivers, and these include pollution emissions, resource extraction. Pressures affect natural processes and manifest themselves in changes in the environmental state (S), which includes abiotic conditions of soil, air and water as well as the biotic conditions of the system (from the genetic to ecosystem level). Impacts (I) are the resulting effects of state changes on human and ecosystem health, resource availability and biodiversity. Responses (R) are the measures taken by society to address drivers, pressures, state or impacts. To further clarify these definitions, we viewed primary drivers of all pressures to be population growth and development which manifest in secondary driving forces which are the specific human activities (e.g. aquaculture, resource extraction) causing pressures and impacts (Gari *et al.* 2015). In addition, we included natural drivers and pressures (e.g. wind waves from meteorological processes) in the assessment. Our rationale is that natural systems are subject to natural drivers that can have effects on the

state of the system either singly or in combination with other stressors, thus although the drivers of natural pressures cannot be managed, their consequences may need to be addressed by management (Smith *et al.* 2014). We further note that this analysis is not a complete DPSIR analysis, but rather focused the "D", "P" and "S" of the DPSIR framework. In this chapter, the term "stressor" has been used synonymously with "pressure" while "state" was used interchangeably with "effect" unless otherwise stated.

Spatial mapping

The second step included an analysis of spatial patterns of multiple stressors. We conducted a data search of the list pressures identified in step 2.2.1. The data search focused on publicly-available data sources, namely official GIS web-portals, maps in published reports by national and regional governmental bodies and published literature. We collected all available spatial data at the site-level (i.e. individual lagoon) and landscape-level (i.e. encompassing multiple lagoons). Whenever possible, we collected data of pressure distribution. However, in some cases, pressure data was not available and data of drivers was used as a proxy. For example, vessel wake pressure was not found therefore we collected data of port locations since vessel traffic and thus vessel wake are likely to be higher closer to ports (Silvestri *et al.* 2013). We mapped individual pressures in the QGIS GIS software to visualize and compare their spatial distribution. We overlapped data of pressures (or drivers) with saltmarsh receptor data to identify saltmarsh areas that are potential under the largest multiple stress. Spatial mapping is recognised as an important tool for the organisation and visualisation of data in multiple stressor assessments (e.g. Halpern *et al.* 2008; Coll *et al.* 2011).

A2.3 Results

A2.3.3 Systematic review

The Web of Knowledge search returned a total of 1798 titles. Of these, 33 papers were determined to be relevant (see Appendix Table A1) (i.e. factorial study on saltmarsh vegetation with two or more stressors reporting the significance of stressor interactions). These papers looked at a total of 12 distinct pairs of stressors with a total of 48 tests (a paper can have more than one test if multiple species, other factors or more than two stressors were tested). Salinity, inundation and temperature were the most-studied stressors on saltmarsh vegetation. Only six stressor pairs had more than one test each (see Table 1). Stressor interactions were present in more than half of the tests (n= 27 out of 48). Here we summarize the findings from stressor pairs with more than one test detected.

- Salinity x inundation

Half of all tests (n=24 out of 48) were concerned with the joint effects of salinity and inundation on growth and/or germination. Half of the tests reported significant interactions (n= 12). Overall, increasing salinity was generally detrimental to growth of saltmarsh vegetation and effects of increasing salinity were more limiting than that of increasing inundation (sensitivity varies between species). When interactions were detected, increased inundation tended to amplify the negative effects of increased salinity.

- Salinity x temperature

All 11 tests that examined the interactive effect of salinity and temperature were concerned with the effect of these two stressors on germination of seeds. In general, interactions between these two stressors were significant (n=9 out of 11 tests). Results were quite variable between tests; in one study lower temperatures increased the resilience of seeds to the detrimental effects of higher salinity (Greenwood & MacFarlane 2006) while another study found low temperature to be the primary limiting factor in germination which was worsened by increasing salinities (Heard & Ancheta 2011). One test found strong interactions on germination of seeds which were further modified by the seed colour, whereby the reduced germination of darker seeds with increasing salinity was further reduced by higher temperatures (Khan, Gul & Weber 2001).

- Salinity x nutrient

Three tests investigated the joint effects of salinity and nutrients. High salinity was generally more limiting than nutrient availability. In one test, increased nutrient availability increases *S. patens* biomass growth, but at higher salinities, increased nutrient did not have a positive effect on biomass. It was found that nutrient availability did not have a linear effect on biomass; some nutrient increase is beneficial for growth but over fertilization led to a reduction in growth (Merino, Huval & Nyman 2010). Ryan & Boyer (2012) found significant stressor interactions on multiple assemblage variables in the glasshouse experiment but not in the field experiment. The authors also reported that the joint effect of salinity and nutrient changed competitive interactions in species assemblages due to differential salt tolerance; at high salinities which inhibit nutrient absorption in salt sensitive species like *D. spicata* and *J. carnosa*, more salt tolerant species like *S. pacifica* were better able to use increased nitrogen to increase biomass growth, potentially resulting in reduced species diversity.

- Inundation x nutrients

Four tests were found for this stressor pair and none reported significant interactions between the stressors. Nelson & Zavaleta (2012)examined the joint effect of nitrogen enrichment and increased inundation in a field experiment. Even though the saltmarsh assemblage in the study was already nitrogen-enriched, vegetation reacted positively to nitrogen addition, while increased inundation had no significant effect on biomass. No interaction was found between nutrient and inundation. Bouma *et al.* (2001) also found no significant interaction of inundation and nutrients on seedling shoot biomass for three saltmarsh species.

Table 1 Tally of number of experiments with each stressor pair in the systematic review.

Stressor pair	Interaction detected	Number of tests
Nutrient x grazing	No	2
	Yes	1
Salinity x nutrient	No	1
	Yes	2
Salinity x temperature	No	2
	Yes	9
Salinity X inundation	No	12
	Yes	12
Inundation x nutrient	No	2
Nutrient x invasive	Yes	1
Salinity x invasive	Yes	1
Salinity x metal	Yes	1

A2.3.4 Multiple stressors in Adriatic saltmarsh

Identifying site-specific drivers and pressures of concern

The literature search identified a total of eight drivers which are currently affecting saltmarsh vegetation in the study area (Table 2). These were damming and river alteration, dredging and canal development, extraction of gas and groundwater, vessel traffic, clam dredging, sea level rise, meteorological processes and geomorphic processes. The drivers resulted

in 14 distinct pressures that cause three state changes in saltmarsh vegetation. The drivers, pressure and state changes and links between them are illustrated in Figure 2. Single drivers (e.g. sea level rise) can lead to multiple pressures and state changes in the system; on the other hand, multiple pressures can also converge on a common adverse state change (e.g. multiple drivers causing lateral erosion).

The link between drivers, pressures and state changes in saltmarsh habitats was clearly made in most studies, although saltmarshes were tangential in a few studies, in which case potential state changes were inferred based on processes described. For example, although saltmarsh waterlogging was not discussed at length in Carbognin *et al.* (2009), the physical processes involved in natural and anthropogenic subsidence was clearly described such that it was possible to deduce the potential state change in saltmarsh (increased water logging) as a result of this subsidence. All pressures resulted in one of three groups of state changes: (1) lateral erosion, (2) reduced sediment availability and (3) waterlogging of saltmarsh vegetation. Lateral erosion causes scouring of saltmarsh platforms at the seaward edge, resulting in bank failure and retreat of the saltmarsh edge (Tonelli, Fagherazzi & Petti 2010). Reduced sediment supply can lead to lower vertical growth of the saltmarsh surface as a result of reduced inorganic sediment deposition and reduced belowground organic biomass accumulation (Morris *et al.* 2002; Fagherazzi *et al.* 2013). Waterlogging of saltmarsh vegetation is associated with reduced plant health and vigour if the plants are subject to increased inundation time beyond its optimal inundation regime (Morris, Sundberg & Hopkinson 2013).

Table 2 Drivers, pressures/ stressors and state change/ effects in Adriatic saltmarshes from qualitative review

Driver	Pressure	Potential saltmarsh state change	Studied lagoons (references)
Vessel traffic	Increased vessel waves	Lateral erosion	Marano Grado (Fontolan *et al.* 2012; Silvestri *et al.* 2013); Venice (Amos *et al.* 2004; Zanatta & Rosato 2005)
Meteorological processes	Wind waves	Lateral erosion	Marano Grado (Fontolan *et al.* 2012); Venice (Carniello *et al.* 2005)
SLR	Increased tidal prism	Lateral erosion	Venice (Day *et al.* 1999)
SLR	Increased wave energy	Lateral erosion	Marano Grado (Fontolan *et al.* 2012); Venice (Day JR *et al.* 1998)
Vessel traffic	Sediment resuspension and loss	Reduced sediment availability	Venice (Amos *et al.* 2004)
Clam dredging	Sediment resuspension and loss	Reduced sediment availability	Venice (Pranovi *et al.* 2004; Sarretta *et al.* 2010)
Damming and river alteration	Reduced sediment supply to lagoons	Reduced sediment availability	Venice (Serandrei-Barbero *et al.* 2006)
Dredging and canal development	Removal of dredge spoils	Reduced sediment availability	Venice (Sarretta *et al.* 2010)
Dredging and canal development	Increased flushing and sediment loss	Reduced sediment availability	Venice (Sarretta *et al.* 2010)
Meteorological processes	Wind waves causing sediment resuspension	Reduced sediment availability	Venice (Amos *et al.* 2004; Tambroni & Seminara 2006)
SLR	Increase in sea level relative to saltmarsh elevation	Waterlogging of saltmarsh	Venice Lagoon (Bellafiore *et al.* 2014); All (Torresan 2011)
Damming and river alteration	Reduced sediment supply to replenish beach barriers leading to increased frequency of breach	Waterlogging of saltmarsh	Po Delta (Bondesan 1989)
SLR	Breaching of beach barriers leading to increased water level in back-barrier marshes	Waterlogging of saltmarsh	Po Delta(Bondesan 1989)
Extraction of gas and groundwater	Land subsidence	Waterlogging of saltmarsh	Venice (Carbognin, Teatini & Tosi 2009; Sarretta *et al.* 2010) (Saretta et al. 2010, Carbognin et al. 2009); Po Delta (Bondesan 1989; Carbognin *et al.* 2009)
Geomorphic processes	Land subsidence from auto-compaction or deformation of substratum	Waterlogging of saltmarsh	Marano Grado (Fontolan *et al.* 2012); Venice (Carbognin *et al.* 2009); Po Delta (Carbognin *et al.* 2009; Teatini, Tosi & Strozzi 2011)

Drivers were generally ubiquitous to all saltmarshes in the study area. Venice lagoon appeared to be the most impacted with the largest number of pressures reported in the literature, although this could also be attributed to it being more thoroughly studied. Less data was available for the Po Delta saltmarshes, although natural and anthropogenic subsidence was well-documented at this site (Bondesan 1989; Cencini 1998). Lateral erosion of saltmarsh edges are occurring in both the Marano Grado and Venice lagoons as a result of increased wave energy (both natural wind- and vessel-generated waves), as well as stronger currents due to an increased tidal prism in the lagoons as a result of sea level rise. Both these lagoons are also experiencing reduced sediment supply which is caused by (1) reduced input of sediment from riverine sources due to hydrological alterations, and (2) loss of sediment from the lagoon system due to direct removal of sediment in the form of dredge spoil, sediment re-suspension from the lagoon bottom as a result of mechanical or natural disturbance and transport of suspended sediments out of the lagoon systems by strong ebb currents. All saltmarsh areas are experiencing increased waterlogging due to eustatic sea level rise and land subsidence. Additionally, in the Po Delta increased waterlogging of back-barrier marshes occurs when protective beach barriers are breached.

Spatial mapping

For saltmarsh receptors, multiple datasets were found with varying degrees of resolution and habitat categories (Table 3). As a result the Corrinne vegetation cover map from 2006 (EEA 2010) was used to maintain a standard resolution and accuracy. Available spatial data for stressors included dissolved nitrogen concentrations, ports and marinas and clam dredging location (Figure 3).

Table 3 Data sources and assumptions made for pressure or driver data

Data type	Spatial cover	Source, processing method, assumptions	Link
Saltmarsh receptors	All lagoons	Shapefile of saltmarsh layer from the EEA Corinne biotope 2006	http://www.eea.europa.eu/data-and-maps/data/clc-2006-vector-data-version-3
Boat traffic	All lagoons	Pagine Azzure (2008)location of marinas	
Clam farming	Marano Grado Venice	Sladonja *et al.* (2011)(digitized) Facca *et al.* (2014)(digitized)	 http://www.hindawi.com/journals/tswj/2014/968618/
Dissolved inorganic nitrogen	Venice Marano Grado Po Delta	WebGIS Atlante della Laguna (digitized from "Azoto inorganico disciolto: media triennio 2001-2003 (interpolazione)") ERSA (2006) (digitized from report "Valutazione della qualità ambientale delle lagune di Marano e di Grado") Assumption: Data taken from a single year and assumed to be representative of annual mean values ARPA (2012) Calculated from mean of dissolved nitrate, nitrite and ammonia of the "Monitoraggio delle acque di transizione della Regione Veneto (2012)".	http://cigno.atlantedellalaguna.it/layers/atlante2006%3AAzotoInorgMELa120012003R http://www.ersa.fvg.it/informativa/atti-dei-convegni-ersa/29-10-08-applicazione-della-direttiva-nitrati-in-regione/zone-vulnerabili-da-nitrati-di-origine-agricola-1/relazione-per-lindividuazione-delle-zone-vulnerabili-da-nitrati/allegato-a-alla-dgr-n-1246/valutazione%20qualita%20ambientale.pdf http://www.arpa.veneto.it/temi-ambientali/acqua/file-e-allegati/documenti/acque-di-transizione/Rapporto%20Acque%20di%20transizione%202012.pdf

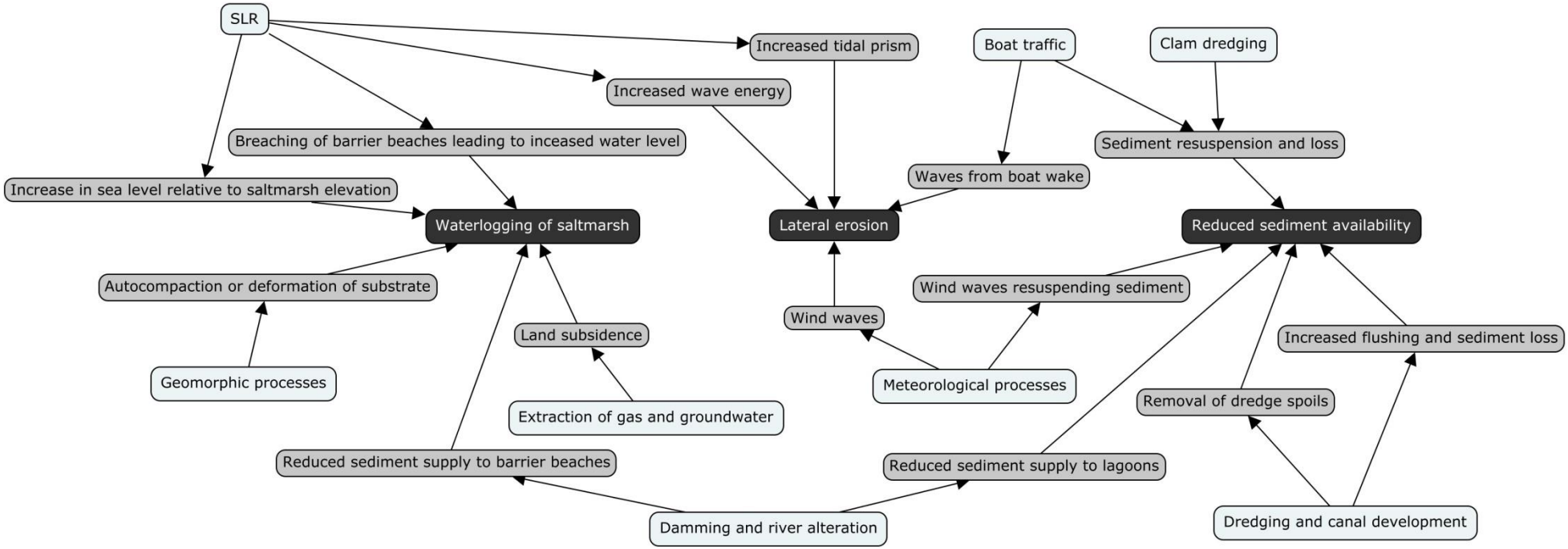

Figure 2 Conceptual model of multiple stressors of saltmarsh vegetation in the Italian Adriatic. Driver, pressure and state change nodes were populated based on a qualitative literature review (see Table 2). Light boxes denote human or natural drivers, grey boxes denote pressures arising resulting from drivers, and black boxes denote state change expected in saltmarshes.

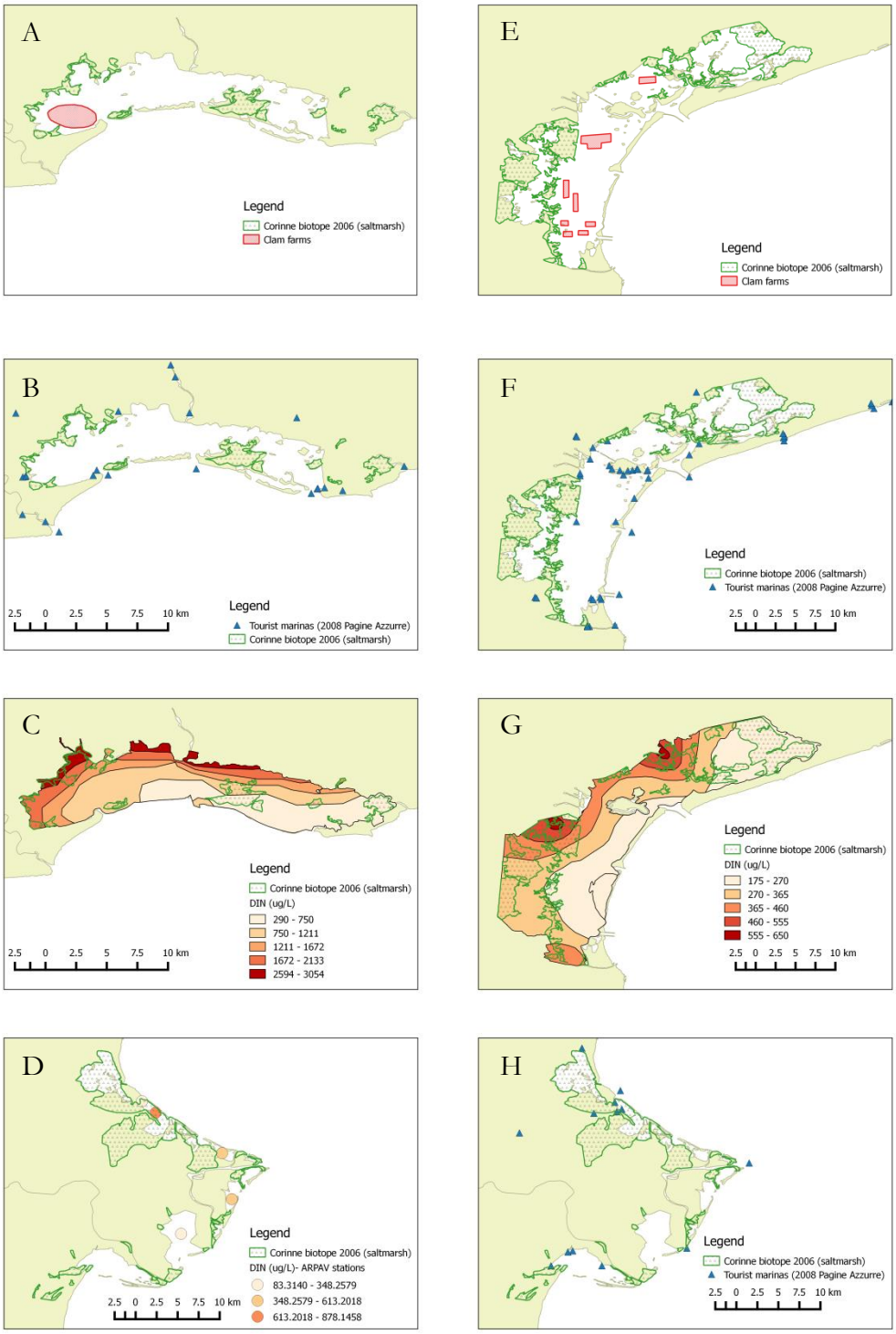

Figure 3 Spatial mapping of pressures (or drivers where pressure data was not available) over saltmarsh receptors in Marano Grado Lagoon (A-C), Venice Lagoon (E-F) and the Po Delta (D, H).

A2.4 Discussion

A2.4.5 Systematic review

In general, the interaction effects varied between tests. However, when interaction was present between salinity and inundation, increased salinity tended to amplify the negative effects of increased inundation. The tests show that response to multiple stressors can be highly case-specific. For example, the interactive effect of two stressors could be further modified by species (Lenssen *et al.* 1993), presence of a third stressor (Baldwin & Mendelssohn 1998), different phenotypes (Khan *et al.* 2001), life stage (e.g. seed germination, growth) (Charpentier, Mesleard & Grillas 1998) and presence of competition (Pennings, Grant & Bertness 2005). This indicates that care should be taken in extrapolating stressor effects based on experiments conducted using other species or in other systems.

A2.4.6 Multiple stressors in the Italian Adriatic saltmarsh literature

This preliminary assessment of multiple stressors has identified key pressures contributing to saltmarsh decline in the Italian Adriatic, and stressors were similar between the three saltmarsh systems to a large extent. Drivers and pressures identified were generally abiotic, modifying the physical processes in the saltmarshes (e.g. increased waterlogging time, increased physical erosional forces and reduced suspended sediment delivery). As expected, the three study systems face co-occurring drivers and pressures, highlighting the need to consider the cumulative effect of stressors when making management decisions.

Some of the stressors are global- or regional- scale stressors (e.g. subsidence and reduced sediment supply) that would be difficult or not feasible to manage. Some other stressors like sediment resuspension from vessels and clam dredging or vessel wake can be adequately managed at the local scale. The Italian Adriatic saltmarshes are under multiple natural and anthropogenic stressors; vessel wake and sediment resuspension can be controlled with speed limit zonations while clam dredging which could perhaps be timed with the tide to cause the least amount of suspended sediment loss. Management of local stressors could alleviate some of the negative effects of regional and global stressors that are truly beyond the sphere of influence of regional and national management bodies (Brown *et al.* 2013).

The literature search found studies of other pressures like increased plastic debris in saltmarsh soil in Venice Lagoon (Vianello *et al.* 2013) and mercury content in saltmarsh soil as a result of industrial pollution in Marano Grado Lagoon (Acquavita *et al.* 2012). However, since there was no clear evidence of a reduction in saltmarsh vegetation growth or condition, these stressors were not further assessed.

A2.4.7 Spatial distribution of multiple stressors in the Italian Adriatic

Collecting sufficient good quality and useful data of stressors was a challenging task. Although there has been a recent push and progress towards making more government data publicly accessible in the EU, large differences exist between countries and regions (Janssen 2011). Overall, the type, quality and accessibility of relevant environmental data varied between the different regions, with data more readily available for the Marano Grado followed by Venice lagoon via the individual regional web-GIS services. Some regions, particularly individual lagoons in the Po Delta yielded relatively little information. Datasets from different sources were often incomparable due to differences in spatial and temporal distribution of measurements, units of measure and data collection technique. In order to be able to identify specific areas within each lagoon that could benefit most from management actions (e.g. areas of highest cumulative stress, most undisturbed areas etc.) data of higher resolution is needed, which was not always the case (e.g. dissolved nitrogen data for the Po Delta saltmarshes).

A2.4.8 Other potential stressors not assessed in Adriatic saltmarsh literature

The systematic review highlighted potentially important stressors that could affect the study systems, but are not yet assessed in the study site. One such stressor is salinity. Half of the tests identified in the systematic review included salinity as a stressor, potentially reflecting the level of influence of this stressor on vegetation condition. Within the ranges tested, increasing salinity had a general negative effect on biomass growth. Salinity in the Marano Grado and Venice lagoons are expected to increasing with future relative sea level rise (Bezzi 2013; Ferrarin *et al.* 2014). This could result in additional stress on saltmarsh plants but has yet to be assessed in this area. The prevalence of interactions between salinity and water level in the systematic review indicate that non-linear effects on saltmarsh could occur as the Adriatic lagoons become increasingly saline and waterlogged as a result of marinisation and relative sea level rise (Bellafiore *et al.* 2014; Ferrarin *et al.* 2014). Since saltmarshes in the study area are already experiencing sub-optimal range of elevations (Kirwan & Megonigal 2013), any increase in waterlogging would result in a negative effect on saltmarsh biomass growth. Increasing salinity (which is a strong limiting stressor) could interact with waterlogging to further reduce saltmarsh growth.

Many lagoons in the study area are affected by chronic nutrient enrichment from agricultural, urban and industrial discharges (Ianni *et al.* 2008; Facca *et al.* 2014). The various lagoons in the Po Delta have a median total dissolved nitrogen concentrations of 80 – 710ug/L

(ARPAV 2012) while the Marano Grado Lagoon has mean total dissolved nitrogen values of up to 3000 ug/L near riverine inputs in the inner lagoon (ARPA FVG 2008). The effect of this chronic nutrient enrichment on saltmarsh vegetation is unknown in the study site. However, recent long-term studies in the USA have found chronic nutrient enrichment to increase rates of lateral erosion as a result of diminished root and rhizome growth and excessive shoot growth (Deegan *et al.* 2012) and reduce soil strength due to increased decomposition of soil carbon (Wigand *et al.* 2009; Turner 2010). This reduction in soil strength at the seaward edge coupled with pressures that lead to lateral erosion could result in an acceleration of edge retreat.

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A2.6 Appendix

Table A1 Systematic review of 48 saltmarsh vegetation multiple stressor tests. Author, data, title, stressors tested and measured response are summarized. Where significant interactions were reported, the associated interaction p-value is shown.

Authors	Year	Title	Factors	Interact	p-value	Response
D. P. J. Kuijper, J. Dubbeld and J. P. Bakker	2005	Competition between two grass species with and without grazing over a productivity gradient	Nutrient x Grazing	No		*E. athericus* biomass
D. P. J. Kuijper, J. Dubbeld and J. P. Bakker	2005	Competition between two grass species with and without grazing over a productivity gradient	Nutrient x Grazing	No		*F. rubra* biomass
T. B. McFalls, P. A. Keddy, D. Campbell and G. Shaffer	2010	Hurricanes, Floods, Levees, and Nutria: Vegetation Responses to Interacting Disturbance and Fertility Regimes with Implications for Coastal Wetland Restoration	Nutrient x Grazing	Yes	p=0.001	Biomass
H.-l. Li, G.-c. Lei, Y.-b. Zhi, S.-q. An, H.-p. Huang, Y. Ouyang, L. Zhao, Z.-f. Deng and Y.-h. Liu	2011	Nitrogen Level Changes the Interactions between a Native (*Scirpus triqueter*) and an Exotic Species (*Spartina anglica*) in Coastal China	Nutrient x Invasive	Yes	p<0.05	Biomass
M. E. Greenwood and G. R. MacFarlane	2009	Effects of salinity on competitive interactions between two *Juncus species*	Salinity x Invasive	Yes	p=0.01	Biomass
S. Redondo-Gomez, L. Andrades-Moreno, E. Mateos-Naranjo, R. Parra, J. Valera-Burgos and R. Aroca	2011	Synergic effect of salinity and zinc stress on growth and photosynthetic responses of the cordgrass, *Spartina densiflora*	Salinity x Metal	Yes	p<0.05	For both tiller and root biomass
A. B. Ryan and K. E. Boyer	2012	Nitrogen further promotes a dominant salt marsh plant in an increasingly saline environment	Salinity x Nutrient	No		Mixed species multiple variables (MANOVA) from field study

Authors	Year	Title	Factors	Interact	p-value	Response
J. H. Merino, D. Huval and A. J. Nyman	2010	Implication of nutrient and salinity interaction on the productivity of *Spartina patens*	Salinity x Nutrient	Yes	p=0.0188	Yes
A. B. Ryan and K. E. Boyer	2012	Nitrogen further promotes a dominant salt marsh plant in an increasingly saline environment	Salinity x Nutrient	Yes	p= 0.015	Mixed species multiple variables (MANOVA) from greenhouse study
M. E. Greenwood and G. R. MacFarlane	2006	Effects of salinity and temperature on the germination of *Phragmites australis*, *Juncus kraussii*, and *Juncus acutus*: Implications for estuarine restoration initiatives	Salinity x Temperature	No		*J. kraussii* germination
M. A. Khan and I. A. Ungar	1998	Germination of the salt tolerant shrub *Suaeda fruticosa* from Pakistan: salinity and temperature responses	Salinity x Temperature	No		Germination
M. E. Greenwood and G. R. MacFarlane	2006	Effects of salinity and temperature on the germination of *Phragmites australis*, *Juncus kraussii*, and *Juncus acutus*: Implications for estuarine restoration initiatives	Salinity x Temperature	Yes	p=0.01	*J. acutus* germination
M. E. Greenwood and G. R. MacFarlane	2006	Effects of salinity and temperature on the germination of *Phragmites australis*, *Juncus kraussii*, and *Juncus acutus*: Implications for estuarine restoration initiatives	Salinity x Temperature	Yes	p= 0.04	*P. australis* germination
S. B. Heard and J. Ancheta	2011	Effects of salinity and temperature on ex situ germination of the threatened Gulf of St. Lawrence Aster, *Symphyotrichum laurentianum Fernald* (Nesom)	Salinity x Temperature	Yes	p=0.012	Germination
M. A. Khan, B. Gul and D. J. Weber	2001	Germination of dimorphic seeds of *Suaeda moquinii* under high salinity stress	Salinity x Temperature	Yes	p=0.001	Germination
M. A. Khan and I. A. Ungar	1997	Germination responses of the subtropical annual halophyte *Zygophyllum simplex*	Salinity x Temperature	Yes	p=0.0001	Germination
B. N. Al-Hawija, M. Partzsch and I. Hensen	2012	Effects of temperature, salinity and cold stratification on seed germination in	Salinity x Temperature	Yes	p=0.01	*A. atripolium* germination

Authors	Year	Title	Factors	Interact	p-value	Response
		halophytes				
B. N. Al-Hawija, M. Partzsch and I. Hensen	2012	Effects of temperature, salinity and cold stratification on seed germination in halophytes	Salinity x Temperature	Yes	p<0.0001	*T. maritimum* germination
B. N. Al-Hawija, M. Partzsch and I. Hensen	2012	Effects of temperature, salinity and cold stratification on seed germination in halophytes	Salinity x Temperature	Yes	p<0.0001	*A. maritima* germination
M. A. Khan, B. Gul and D. J. Weber	2004	Temperature and high salinity effects in germinating dimorphic seeds of *Atriplex rosea*	Salinity x Temperature	Yes	p<0.001	Final germination
J. B. Adams and G. C. Bate	1995	Ecological implications of tolerance of salinity and inundation by *Spartina maritima*	Salinity x Inundation	No		Stem elongatoion
A. H. Baldwin and I. A. Mendelssohn	1998	Effects of salinity and water level on coastal marshes: an experimental test of disturbance as a catalyst for vegetation change	Salinity x Inundation	No		Undisturbed community biomass
R. J. Howard and P. S. Rafferty	2006	Clonal variation in response to salinity and flooding stress in four marsh macrophytes of the northern gulf of Mexico, USA	Salinity x Inundation	No		*P. australis* biomass
R. J. Howard and P. S. Rafferty	2006	Clonal variation in response to salinity and flooding stress in four marsh macrophytes of the northern gulf of Mexico, USA	Salinity x Inundation	No		*S. californicus* biomass
R. J. Howard and P. S. Rafferty	2006	Clonal variation in response to salinity and flooding stress in four marsh macrophytes of the northern gulf of Mexico, USA	Salinity x Inundation	No		*S. robustus* biomass
G. M. Lenssen, W. E. Vanduin, P. Jak and J. Rozema	1995	The response of *Aster tripolium* and *Puccinellia maritima* to atmospheric carbon-dioxide enrichment and their interactions with flooding and salinity	Salinity x Inundation	No		Biomass
S. B. Martin and G. P. Shaffer	2005	*Sagittaria biomass* partitioning relative to salinity, hydrologic regime, and substrate	Salinity x Inundation	No		Biomass

Authors	Year	Title	Factors	Interact	p-value	Response
		type: Implications for plant distribution patterns in Coastal Louisiana, United States				
G. Naidoo and J. Kift	2006	Responses of the saltmarsh rush *Juncus kraussii* to salinity and waterlogging	Salinity x Inundation	No		Biomass
S. C. Pennings, M. B. Grant and M. D. Bertness	2005	Plant zonation in low-latitude salt marshes: disentangling the roles of flooding, salinity and competition	Salinity x Inundation	No		*J. roemerianus* biomass
C. H. Wang, M. Lu, B. Yang, Q. Yang, X. D. Zhang, T. Hara and B. Li	2010	Effects of environmental gradients on the performances of four dominant plants in a Chinese saltmarsh: implications for plant zonation	Salinity x Inundation	No		
I. Woo and J. Y. Takekawa	2012	Will inundation and salinity levels associated with projected sea level rise reduce the survival, growth, and reproductive capacity of *Sarcocornia pacifica* (pickleweed)?	Salinity x Inundation	No		Adult biomass
I. Woo and J. Y. Takekawa	2012	Will inundation and salinity levels associated with projected sea level rise reduce the survival, growth, and reproductive capacity of *Sarcocornia pacifica* (pickleweed)?	Salinity x Inundation	No		Seedling biomass
A. H. Baldwin and I. A. Mendelssohn	1998	Effects of salinity and water level on coastal marshes: an experimental test of disturbance as a catalyst for vegetation change	Salinity x Inundation	Yes	$p<0.0001$	Disturbed community biomass
A. Charpentier, F. Mesleard and P. Grillas	1998	The role of water level and salinity in the regulation of *Juncus gerardi* populations in former ricefields in southern France	Salinity x Inundation	Yes	$p<0.05$	Shoot biomass
J. G. Engels, F. Rink and K. Jensen	2011	Stress tolerance and biotic interactions determine plant zonation patterns in estuarine marshes during seedling emergence and early establishment	Salinity x Inundation	Yes	$p<0.001$	Halophyte seedling emergence
R. J. Howard and P. S. Rafferty	2006	Clonal variation in response to salinity and flooding stress in four marsh	Salinity x Inundation	Yes	$p=0.0018$	*D. spicata* biomass

Authors	Year	Title	Factors	Interact	p-value	Response
		macrophytes of the northern gulf of Mexico, USA				
S. C. Pennings, M. B. Grant and M. D. Bertness	2005	Plant zonation in low-latitude salt marshes: disentangling the roles of flooding, salinity and competition	Salinity x Inundation	Yes	p= 0.048	*S. alterniflora* biomass
E. C. Webb and I. A. Mendelssohn	1996	Factors affecting vegetation dieback of an oligohaline marsh in coastal Louisiana: Field manipulation of salinity and submergence	Salinity x Inundation	Yes	p<0.05	Biomass
R. A. Konisky and D. M. Burdick	2004	Effects of stressors on invasive and halophytic plants of New England salt marshes: A framework for predicting response to tidal restoration	Salinity x Inundation	Yes	p=0.03	*S. alterniflora* relative shoot biomass growth
R. A. Konisky and D. M. Burdick	2004	Effects of stressors on invasive and halophytic plants of New England salt marshes: A framework for predicting response to tidal restoration	Salinity x Inundation	Yes	p=0.0001	*S. patens* relative shoot biomass growth
R. A. Konisky and D. M. Burdick	2004	Effects of stressors on invasive and halophytic plants of New England salt marshes: A framework for predicting response to tidal restoration	Salinity x Inundation	Yes	p=0.0212	*J. gerardii* relative shoot biomass growth
R. A. Konisky and D. M. Burdick	2004	Effects of stressors on invasive and halophytic plants of New England salt marshes: A framework for predicting response to tidal restoration	Salinity x Inundation	Yes	p=0.0024	*P. australis* relative shoot biomass growth
R. A. Konisky and D. M. Burdick	2004	Effects of stressors on invasive and halophytic plants of New England salt marshes: A framework for predicting response to tidal restoration	Salinity x Inundation	Yes	p=0.0058	*T. angustifolia* relative shoot biomass growth
C. E. Brown and S. R. Pezeshki	2007	Threshhold for recovery in the marsh halophyte *Spartina alterniflora* grown under the combined effects of salinity and soil drying	Salinity x Inundation	Yes	p<0.0001	Biomass
T. J. Bouma, B. P. Koutstaal, M. van	2001	Coping with low nutrient availability and inundation: root growth responses of	Inundation x Nutrient	No		Shoot dry weight for *E. pycnanthus*

Authors	Year	Title	Factors	Interact	p-value	Response
Dongen and K. L. Nielsen		three halophytic grass species from different elevations along a flooding gradient				
T. J. Bouma, B. P. Koutstaal, M. van Dongen and K. L. Nielsen	2001	Coping with low nutrient availability and inundation: root growth responses of three halophytic grass species from different elevations along a flooding gradient	Inundation x Nutrient	No		Shoot dry weight for *P. maritima*
T. J. Bouma, B. P. Koutstaal, M. van Dongen and K. L. Nielsen	2001	Coping with low nutrient availability and inundation: root growth responses of three halophytic grass species from different elevations along a flooding gradient	Inundation x Nutrient	No		Shoot dry weight for *S. anglica*
J. L. Nelson and E. S. Zavaleta	2012	Salt marsh as a coastal filter for the oceans: changes in function with experimental increases in nitrogen loading and sea-level rise	Inundation x Nutrient	No		Both above and belowground biomass

Article 3 Nutrient levels modify saltmarsh responses to increased inundation in different soil types

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Abstract

Saltmarshes have been depleted historically, and cumulative stressors threaten their future persistence. We examined experimentally how nutrient availability (high vs. low) affects the responses of *Spartina maritima* to increased inundation in two mineral soil types (low vs. medium organic). Increased inundation, one effect of accelerated sea level rise, had negative effects on most plant growth parameters, but the magnitude varied with soil and nutrient levels, and between plants from different locations. Average differences between inundation treatments were strongest at high nutrient conditions in low organic matter soils. We conclude that saltmarsh vegetation would be more drastically affected by increased inundation in low than in medium organic matter soils, and especially in estuaries already under high nutrient availability. This knowledge enhances the prediction of changes at the foreshore of saltmarshes related to sea level rise, and the development of site-specific conservation strategies.

Keywords: *Spartina maritima*, saltmarsh, multiple stressors, marsh organ, inundation, nutrients

A3.1 Introduction

Many valuable coastal ecosystems have declined due to the cumulative effects of local and global anthropogenic stressors (Halpern *et al.* 2008; Ban *et al.* 2013; Strain *et al.* 2014), leading to loss of valuable ecosystem services and contributing to persistent poverty and risk (Millennium Ecosystem Assessment 2005). Over the centuries, coastlines have been altered by land reclamation and development, overfishing, pollution and species invasions (Lotze *et al.* 2006; Airoldi & Beck 2007). When coupled with climatic instabilities, localized human perturbations are creating new disturbance regimes which are further accelerating the degradation and decline of coastal ecosystems (Claudet & Fraschetti 2010). With current delays and limitations on the mitigation of global stressors, it is crucial to identify which factors can maintain ecosystem resilience at local scales (Brown *et al.* 2013).

Saltmarshes are prime examples of a threatened coastal ecosystem that supports important ecosystem services including maintenance of water quality, provision of habitat, carbon sequestration and control of shoreline erosion (Zedler & Kercher 2005; Ysebaert et al. 2011; Silliman et al. 2012). Saltmarshes globally have been in steady decline (Lotze et al. 2006), with reductions in Europe by more than 60% (Airoldi & Beck 2007) and in the US by 50% (Kennish 2001). Remaining tracts of intact saltmarshes are threatened by a variety of human-related stressors (e.g. Silliman & Bertness 2004; Goldman Martone & Wasson 2008; Uhrin & Schellinger 2011; Lin & Mendelssohn 2012) including two ubiquitous factors impacting coastal zones worldwide: excess nutrient input, a local scale stressor arising from intensive farming and inadequate wastewater treatment in the watershed, and relative sea level rise (RSLR) accelerated by climate change and amplified in some regions by land subsidence (FitzGerald et al. 2008; Deegan et al. 2012).

Relative sea level rise affects saltmarsh biogeochemistry through a variety of processes. As sea level rises relative to the existing saltmarsh elevation, inundation time of the saltmarsh platform increases. Increased inundation inhibits plant growth due to a combination of mechanisms, including reduced photosynthesis related to stomatal closure, root death due to anoxia, and reduced growth resulting from build-up of toxic compounds in the soil (e.g. hydrogen sulphide) under low redox and anaerobic conditions (DeLaune, Pezeshki & Patrick 1987). In addition, reduced root biomass production hampers the ability of saltmarshes to grow vertically to keep up with RSLR (Morris *et al.* 2002; DeLaune & Pezeshki 2003; FitzGerald *et al.* 2008), while increased root death and subsequent loss of root turgor can result in peat collapse which further enhances vertical elevation loss in a detrimental feedback loop (DeLaune *et al.* 1994). Consequently, increased inundation can

disproportionately reduce belowground biomass production, leading to lower root-to-shoot (R:S) ratio (Janousek & Mayo 2013). Further, reduced shoot production hampers aboveground sediment trapping and accretion, the second mechanism by which saltmarshes maintain their vertical position relative to the tidal frame (Morris *et al.* 2002). However, these mechanisms may also be counteracted by the hydrological effect of increased sediment deposition rates arising from the higher inundation depths and longer inundation times (Fagherazzi *et al.* 2012). Increased inundation is just one of several processes related to RSLR that can potentially negatively affect saltmarshes; other effects include higher wave erosion at the saltmarsh edge due to the deepening and enlarging of tidal bays (Fagherazzi *et al.* 2013) and increased deposition of suspended sediment on the saltmarsh platform reducing sediment supply to the saltmarsh edge and thus reducing the saltmarsh's ability to withstand lateral erosion (Mariotti & Fagherazzi 2013). Inability of saltmarsh biomass accumulation and sediment accretion to keep pace with increasing sea levels will result in loss of high/mid marshes and conversion of low marshes to mudflats (Schile *et al.* 2014).

Nutrient enrichment affects estuaries and coastal waters globally (Selman *et al.* 2008). On one hand, saltmarshes are recognized as important nutrient sinks, mediating the effects of excess nutrients, particularly nitrogen (Craft 1996). Nitrogen-cycling is generally so efficient in saltmarshes that marsh ecosystems tend to be nitrogen-limited (Valiela *et al.* 1978; Mendelssohn 1979; Kiehl, Esselink & Bakker 1997; Boyer *et al.* 2001; Crain 2007). Excess nitrates are removed by assimilation into primary production, denitrification (Mitsch & Gosselink 2000; Wigand *et al.* 2009), and to a much lower extent by bacterially-mediated anaerobic ammonia oxidation (Koop-Jakobsen & Giblin 2009). On the other hand, concerns are being raised that increased nutrient availability to saltmarshes could be facilitating saltmarsh loss (Deegan *et al.* 2012). Some evidence shows that high nitrogen availability can suppress belowground biomass production and accumulation and perhaps increase organic matter decomposition, resulting in loss of soil stability, increased soil erosion and saltmarsh habitat loss (Turner *et al.* 2009; Turner 2010; Deegan *et al.* 2012; Watson *et al.* 2014). With respect to the effect of nutrient enrichment on root biomass, short- and long-term mesocosm and field experiments have produced mixed responses in saltmarsh vegetation (Table 1). Nutrient enrichment reduced root biomass growth in most studies, while increased or unaffected growth has also been reported. Furthermore, the direct and indirect effects of elevated nutrient availability on saltmarsh soil has also been mixed (Table 4 and review by Morris, Shaffer & Nyman 2013). These mixed results in vegetation responses most likely relate to differences in the magnitude of nutrient enrichment (Valiela, Teal & Persson 1976) and species types (e.g. Ravit *et al.* 2007; Adam Langley *et*

al. 2013).

Since nutrient cycling occurs in a large part in saltmarsh sediments, the local soil conditions could significantly modify the effects of nutrient enrichment. Saltmarshes can be found on a variety of soils, ranging from mineral-dominated to organic-dominated (Mitsch & Gosselink 2000). Studies comparing mature saltmarshes with recently formed saltmarshes (both natural and man-made) report that the soil bulk density tends to decrease while particulate organic matter, soil carbon and nitrogen content increase as saltmarshes mature (Craft, Broome & Seneca 1988; Osgood & Zieman 1993; Craft 2000; Havens, Varnell & Watts 2002; Edwards & Proffitt 2003). As far as we are aware, no studies have empirically addressed how saltmarshes of different soil types and with different nutrient availability respond to increased inundation.

We conducted a field experiment to investigate the response of low intertidal saltmarsh growth to increased inundation in different soil types and nutrient availabilities. We chose the nutrient levels based on realistic nutrient conditions of lagoons in the study region while the high inundation level simulated a sea level increase of 10cm which has been estimated to occur close to year 2020 based on sea level scenarios developed for the Italian North Adriatic coastline (THESEUS 2009). We chose two soil types that differed in the amount of organic matter content, reflecting respectively emergent or restored marshes with lower organic content and mature marshes with higher organic content. We predicted that (i) saltmarsh vegetation will be negatively affected by an increase in inundation as a result of a 10 cm simulated increase in water level relative to current sea level, (ii) low nutrient conditions will help to maintain greater root biomass and R:S ratios thereby mitigating the negative effects of higher inundation, and (iii) the effects of higher nutrients on root growth will be dampened in soils with higher organic content due to its ability to support higher rates of denitrification. We focused on *Spartina maritima* (hereby referred to as *Spartina*) as this is the most relevant foundation species in the low intertidal saltmarsh vegetation in the study region.

Table 1 Effects of higher nutrient availability on shoot and root biomass, R:S biomass ratio and soil, summarized from enrichment experiments and correlational studies. ↑: increase, ↓: decrease, ↔: no change, NR: not reported. * Trend estimated from reported data.

Reference	Species and study duration	Shoot biomass	Root biomass	R:S biomass ratio	Soil effects	Suggested mechanism
Valiela *et al.* (1976)	*S. alterniflora* & *S. patens*, 7 months	↑	↓at highest nutrient concentrations	↓*	NR	Some grasses stop producing more roots once N absorption is adequate.
Morris & Bradley (1999)	*S. alterniflora*, 12 years	↓	NR	NR	↓ soil C stores; ↑ sediment accretion	Soil C loss from increased soil respiration; dense shoots trap more sediment.
Boyer *et al.* (2000)	*S. foliosa*, 2 years	NR	↔	NR	NR	Low N-retention in soil due to sandy substrate or poor conversion rate by microorganisms.
Wigand *et al.* (2004)	*S. patens*, 2.5 years	↑	↔	↓	NR	Removal of N-limitation allows plants to shift energy to shoot growth, requiring additional P, which can be achieved via stimulation of endomycorrhizal colonization in existing roots, instead of increasing root density.
Tyler *et al.* (2007)	(a) Invasive hybrid *Spartina* (*S. foliosa* x *S. alterniflora*),	↑	↔	↓at edge plots	NR	Root biomass in this eutrophic location likely not N-limited.

Reference	Species and study duration	Shoot biomass	Root biomass	R:S biomass ratio	Soil effects	Suggested mechanism
	San Francisco Bay, 5 months					
	(b) Invasive *S. alterniflora,* Willapa Bay, 5 months	↑	↑ in meadow plots	↓at edge plots	NR	Root biomass in this location is N-limited so N-addition led to increased root growth. However, shoot biomass increased proportionately more than root biomass.
Darby & Turner (2008b)	*S. alterniflora*, 5 months	↑	↔ when only N added; ↓ when N+P added	↓ when either N or P added	↓ in soil redox potential	N-addition stimulated shoot biomass only; root foraging relaxed when P-availability increased.
Turner *et al.* (2009)	*S. alterniflora, S. patens, Disticlis spicata* mixed marsh, 36 years	↑	NR	↓	↔ in soil accumulation rate; organic matter accumulation ↓ in deep soil layer but ↔ in upper layer; ↓ shear vane strength	Increase in carbon loss via denitrification and other coupled processes between C metabolism and N cycle.
Wigand *et al.* (2009)	*S. alterniflora, S. patens*	NR	↓ for S. patens	NR	↑ in soil respiration rate	Increased turnover of labile C and N in upper layer of *S. alterniflora* marsh.

Reference	Species and study duration	Shoot biomass	Root biomass	R:S biomass ratio	Soil effects	Suggested mechanism
	(correlation data)				for both species	
Anisfeld & Hill (2012)	*S. alterniflora*, 5 years	↑	↔	↓*	↔ in soil C; ↔ in elevation; slight ↑ in accretion in N + P fertilized plots	Loss of soil C in high nutrient treatment is offset by other processes, e.g. increased gross root production and respiration, leading to no change in belowground primary production.
Nelson & Zavaleta (2012)	*Sarcocornia pacifica, Jaumea carnosa, Frankenia salina,* and *D. spicata* (perennial), 2 years	↑	↑	↓*	NR	Shoot biomass increase was much larger than root biomass increase; capacity to absorb N remains even in "hypertrophic" environment.
Deegan *et al.* (2012)	S. alterniflora and S. patens, 9 years	↑	↓	↓	↓ soil and creek bank stability;	Faster decomposition of N-rich leaf detritus; increased denitrification and soil respiration accelerated the decomposition

Reference	Species and study duration	Shoot biomass	Root biomass	R:S biomass ratio	Soil effects	Suggested mechanism
					conversion of marsh to open water	of soil C, leading to higher fine organic matter and water content in soil; reduced root stabilising role coupled with taller shoots with weaker structure and wetter soil caused loss of soil structural integrity.
Fox *et al.* (2012)	Various, 30+ years	General shift from *S. alterniflora* to *D. spicata* dominated; ↑ where *S. alterniflora* remained	NR	NR	↑ elevation in areas where *D. spicata* became dominant	Nutrient enrichment led to species change from *S. alterniflora* to *D. spicata* dominant; elevation increase in *D. spicata* areas due to higher biomass accumulation, lower decomposition rate and increased mineral sediment trapping.
Langley *et al.* (2013)	*Schoenoplectus americanus* & *S. patens*, 2 years	↑	↑	↔	NR	No reduction in root foraging even with nutrient addition due to severe N-limitation
Watson *et al.* (2014)	*S. alterniflora*, 81 days	↔	↔ in biomass but shift in root morphology from coarse to finer roots	NR	↑ decomposition and sulfide production	Nutrient enrichment under anoxic conditions increased organic matter decomposition resulting in increased sulfide concentration and higher CO_2 flux.

A3.2 Methods

A3.2.1 Study area

The Po Delta is located in the northern Adriatic and is bordered by the Venice Lagoon to the north and sandy beaches of the Romagna to the south. The delta and adjoining coasts are characterised by low relief and shallow coastal wetlands and beaches and experience an average tidal range of 0.8m while tides up to > 1.8m can occur during extreme storm surges. A large part of the delta lies below sea level and is defended by numerous coastal protection structures (Cencini 1998). Saltmarshes in this region have one of the longest history of degradation and conversion (Airoldi & Beck 2007). While direct conversion is now restricted, saltmarshes are faced with other continuing human impacts, including excessive nutrient input from intensive farming, industry and dense urban centres in the watershed (Nespoli 1988) and RSLR driven both by global increasing sea level and localised land subsidence (Cencini 1998). Pockets of extensive coastal saltmarshes remain, but growth dynamics and basic ecological processes are poorly known (Scarton, Day & Rismondo 2002).

Spartina plugs used in the mesocosm experiment were collected from two lagoons; Vallona Lagoon and Bellocchio Lagoon. Vallona Lagoon (45°01'40.8"N, 12°23'01.4"E) is located in the current-day Po Delta. The lagoon was created unintentionally in the 1970's when previously drained farmland was re-flooded by seawater due to severe land subsidence. Like the rest of the coast, Vallona Lagoon experiences a semi-dirunal micro-tidal regime (Consorzio di Bonifica Delta del Po 2013). It is used privately for shellfish aquaculture and seasonal hunting, and has sluice gates that are actively managed to maximize water exchange and maintain good water quality (Fondazione Ca' Vendramin 2010), such that compared to other lagoons in the Veneto region, it has a moderate to low level of nitrate (Appendix S1). Bellocchio Lagoon (44°37'39.8"N, 12°15'55.8"E) is located about 50km south of Vallona Lagoon in the fossil Po Delta which is no longer part of the active delta. The latter lagoon is a back-barrier lagoon behind an active sand dune and is connected to the sea by a channel that is maintained by periodic dredging. Bellocchio Lagoon is one of few lagoons with low nitrate levels in the region (Lo *et al.* unpublished data). Both lagoons are small and sheltered by natural or man-made barriers, but are freely influenced by tides. The low intertidal saltmarsh assemblage is similar at both lagoons, predominantly comprising of mono-specific tussocks of *Spartina* interspersed with *Salicornia veneta* and bare soil at an elevation of 0.1 to 0.3m above mean sea level (a.m.s.l.). *Spartina* is a perennial grass growing on a variety of substrate types ranging from silt to sand and peat. In the Vallona Lagoon, *Spartina* has created patches of organic-rich soil in the

parent substrate, which is predominantly mineral soil. The same species also dominate the remaining saltmarshes in the region. Information on the geomorphology, hydrology and environmental characteristics of the two lagoons of the region can be found in Villatoro *et al.* (2014) and Fondazione Ca' Vendramin (2012).

A3.2.2 Experimental design and setup

The mesocosm experiment was carried out in Vallona with plugs from both lagoons. The mesocosm was not replicated in Bellocchio Lagoon to avoid impacts to the small and fragile natural ecosystems, which are protected as a Natura 2000 Site of Community Interest. In July 2013, we simulated eight scenarios with orthogonal combinations of two inundation levels (low vs. high inundation), two nutrient levels (low vs. high nutrient conditions) and two soil types (low organic matter LOM vs. medium organic matter MOM). Each scenario was replicated eight times (n=8), for a total of 64 units. We planted half the units with *Spartina* plugs from Vallona and the other half with plugs from Bellocchio. Plugs with shoots and roots were collected from the edge of *Spartina* colonies and were thinned with scissors to the soil surface (Pennings *et al.* 2005) until five shoots of 10-15cm height remained. We ran the experiment for three months over the main growing and flowering period, i.e. July - September.

Experimental units consisted of round PVC-pipes (16cm diameter, 40cm height), hereby referred to as a pot. Each pot comprised an open top and closed bottom lid. We drilled eight holes in each lid to facilitate vertical water movement and lined the bottom with a nylon mesh to prevent mixing of the soil within the pots with the surrounding native soil. Each pot was pushed into the substrate until the top of the pot reached the designated height; we then secured the pots with cable ties to three 1.5m poles hammered into the surrounding substrate to prevent movement during storms.

The pots were equally distributed in two parallel rows 1.5m apart (Figure 1). We manipulated inundation time by varying the height of the pots. These pots are a modification of the "marsh organ" technique (Morris 2007) in that individual pots are not linked to other pots and have a bottom cap and thus fixed volume of soil. The "low" inundation level was defined as the average tidal level at which *Spartina* is currently growing at the experimental site (0.1 a.m.s.l.) while the "high" inundation was set 10cm lower (0 a.m.s.l.). Based on sea level data from the ISPRA regional monitoring programme (http://www.venezia.isprambiente.it) from the northern Adriatic as well as tidal level data loggers deployed at Bellocchio Lagoon, a decrease in elevation of 10cm corresponds

approximately with a 20% increase in inundation time.

Figure 1
Layout of the experiment at Vallona Lagoon. Pots were arranged in two rows to different inundation levels (low vs. high). Each row contained four replicates for each combination of two nutrient treatments (high vs. low conditions) and two soil types (low organic matter, LOM vs. medium organic matter, MOM) for a total of 32 pots per row.

Soil type and nutrient levels were imposed orthogonally at random to each of the inundation regimes (Figure 1). We collected the two soil types from within a 200m radius of the experimental site in the intertidal zone. The LOM substrate was taken from un-vegetated areas while the MOM substrate from vegetated areas patchily interspersed within the intertidal zone. At the start of the experiment, we collected four replicate soil samples from each of the two soils types for analyses of macro-organic matter content, organic matter content (using the Loss on Ignition (LOI) method) and soil bulk density. For each replicate sample, we washed 30ml of sample over a 2mm mesh diameter sieve to separate out the macro-organic matter which was dried at 70°C and weighed. Then, we weighed and dried another 20ml of sample at 70°C to constant weight to obtain the bulk density. We ignited a sub-sample from the bulk density analysis at 450°C for 4h to estimate the organic matter ash free dry weight (Curado *et al.* 2013). The LOM treatment consisted of sediment with low organic content (2.7% ± 0.49) and high bulk density (1.3 ± 0.05 g/cm^3) while the MOM treatment had higher organic content (10.0% ± 0.96 g/cm^3) and low bulk density (0.1 ± 0.07 g/cm^3) (Table 2). Both soil types were predominantly fine sand (125-250μm) with a total sand content (62.5μm - 1.6mm) of approximately 80%. After filling with the assigned soil type, the pots were left

for two weeks to allow for soil settlement before the experiment. At the start of the experiment, each plot was planted with one plug and re-filled to the top with the assigned soil type.

We selected nutrient treatment levels to reflect realistic nitrate concentrations in coastal lagoons monitored by the Veneto Regional Environmental Protection Agency (see Appendix S1 for details). The existing nitrate level of Vallona Lagoon was chosen as the low nutrient scenario while the high nutrient level was based on the nearby Caorle Lagoon (Figure S1.1) which records some of the highest nitrate concentrations in the region due to a combination of factors, including reduced water exchange and land-based runoff (Fondazione Ca' Vendramin 2010). We achieved the high nutrient level with the slow-release fertilizer Osmocote ® Exact Standard NPK. Pre-tests indicated that a dose of 45g/pot achieved a soil pore water nitrate-N concentration of approximately 1.5mg/L (Appendix S1, Figure S1.2).

Table 2 Properties of the two experimental soil types. Data are mean and SE over four replicates. For details see methods.

	Low organic matter (LOM)		**Medium organic matter (MOM)**	
	Mean	S.E.	Mean	S.E.
Bulk density (g/cm^3)	1.3	0.05	0.1	0.07
Organic matter (%)	2.7	0.49	10.0	0.96
Macro-organic matter (%)	0.6	0.22	4.7	2.35
Sand (%)	80.3	2.06	78.2	3.72
Silt (%)	17.9	1.89	19.8	3.40
Clay (%)	1.9	0.20	2.0	0.32

A3.2.3 Measured response variables

At the start of the experiment, we set aside ten reference *Spartina* plugs from each donor site for analysis of live shoot and root biomass. We clipped the live shoots at the soil surface and washed root clumps carefully over a 1mm sieve to remove all sand and silt while large organic and inorganic debris were removed by hand. Live roots were distinguished from dead roots and other dead organic

matter by colour, texture and density (Darby & Turner 2008). Live dry biomass for roots and shoots were obtained after drying separately at 70°C to constant weights, and then weighed to the nearest 0.1g.

Four replicates were lost immediately after the set-up as a strong storm washed away the transplanted plants. Those pots were removed from the calculations; therefore, the number of available replicated pots varied between three and four. At the end of the experiment, we estimated percentage survival of plants per treatment as number of pots with living *Spartina* over number of available pots. In pots with remaining *Spartina* (live and dead) at the end of the experiment, we estimated soil loss by measuring the distance of the soil surface within each pot relative to the top of the pot at four haphazard positions. We took four redox readings from each pot for the first 2mm surface soil with a handheld probe (Hanna Instruments HI 8314 with redox probe HI 3230).

All live plants were then extricated carefully from the pots and brought to the lab for the following measures: number of live shoots, height of the five tallest live shoots and number of flowers per pot. After these measures were taken, we analysed the samples for live shoot and root biomass following the procedure used for the reference *Spartina* plugs at the start of the experiment. We obtained the R:S ratio by dividing live root dry biomass by live shoot dry biomass. We analysed representative samples of root and shoot biomass from each pot for elemental carbon (C) and nitrogen (N) composition (Organic Elemental Analyzer Flash 2000, Interscience). Dead plants were scored as having zero root and shoot biomass and zero number of shoots and flowers, while tallest live shoot height, C and N content and R:S ratio were considered as missing values.

A3.2.4 Statistical analysis

While all *Spartina* from Bellocchio survived to the end of the experiment and showed changes in tissue N content consistent with the nutrient manipulation, the plugs from Vallona experienced high mortality, leading to lack of sufficient numbers of live plants for statistically meaningful measurements (see Results). Therefore, plants from Vallona were not further analysed.

The responses of *Spartina* from Bellocchio to inundation in different nutrient environments and soil types were analysed using permutational ANOVAs with three fixed orthogonal factors: inundation (low vs. high), nutrient level (low vs. high), and soil type (LOM vs. MOM). The variables analysed were: number of shoots, number of flowers, mean height of tallest live shoots, live shoot and root biomass, R:S biomass ratio, shoot and root C:N ratio, and soil loss. We used the statistical procedure PERMANOVA in PRIMER (Anderson, Gorley & Clarke 2008) to partition the

variability and obtain F-statistics on matrices of Euclidean distances calculated from the original raw data. P-values were calculated using 9999 random permutations of the appropriate exchangeable units and Type III sums of squares under the reduced model. All analyses began with a full model, with all possible interactions included; a final simplified model was selected by sequentially dropping highly non-significant interaction terms (P>0.25) following Underwood (1996) and Anderson *et al.* (2008). Statistical results for the full models are presented in Appendix S2. We used permutational ANOVA rather than the classic ANOVA test due to ease of use with the slight unbalanced number of replicates (3 to 4) arising from one missing pot. All variables were tested for homogeneity of dispersion between groups with the PERMDISP function in PERMANOVA. No significant dispersion was detected.

A3.3 Results

All plants from Bellocchio survived to the end of the experiment (Table 3). Plugs increased from the initial five shoots to an average of 44 ± 9.6 shoots per pot (Figure 2), and produced on average 2 ± 0.4 flowers per pot. The CHN analysis revealed percentage N and C: N ratios of the roots to be significantly higher and lower, respectively, in the high than low nutrient treatments; this was consistent with the experimental nutrient manipulation (see Appendix S1 for details). Thirty-three percent of plants from Vallona died (Table 3), with the highest mortality in the increased inundation treatments in LOM soils. The surviving plants from Vallona did not grow nor produced flowers (Appendix S3) and tissue N content did not seem to show differences in nutrient uptake between treatments (Appendix S1). Because of insufficient numbers of live plants for statistically meaningful replicated measurements, and no evidence of different nutrient uptake between treatments, plants from Vallona were excluded from further analyses, and only data from Bellocchio are presented. The data collected from the surviving plants of Vallona are reported in Appendix S3 for completeness of results.

The effects of increased inundation on *Spartina* varied with both nutrient levels and soil types (Figure 2). High inundation resulted in lower mean shoot and root biomass and number of flowers; the largest decreases in these plant measures compared to low inundation occurred in the high nutrient with LOM soil treatments (significant or marginally significant inundation x nutrient x soil interactions, Table 4). Number of shoots and mean tallest shoot heights also decreased between low

and high inundation levels; these differences were largest at high nutrient conditions (significant or marginally significant inundation x nutrient interactions, Table 4), while soil type did not lead to significant effects (Figure 2, Table 4). Treatments did not result in significant differences in soil redox potential (Table 4).

High inundation disproportionately affected root and shoot biomass resulting in significant effects on R:S ratios. Increased inundation decreased biomass growth in both shoots and roots, but the effects were more severe for shoots, leading to overall higher R:S ratios under high inundation conditions compared to low inundation treatments. Lower nutrient condition lead to higher R:S ratios across both inundation levels and soil types, with the highest R:S ratio found in the low nutrient combined to LOM soil treatment, but these trends were not significant (Table 4). MOM pots lost on average 1.6 ± 0.35cm and 2.6 ± 0.29cm of soil relative to the top of the pots in low and high inundation treatments respectively, while no differences with inundation were observed for LOM pots, which lost on average 1.9 ± 0.13cm of soil (significant inundation x soil interaction, Table 4). No differences in soil loss were observed between nutrient treatments.

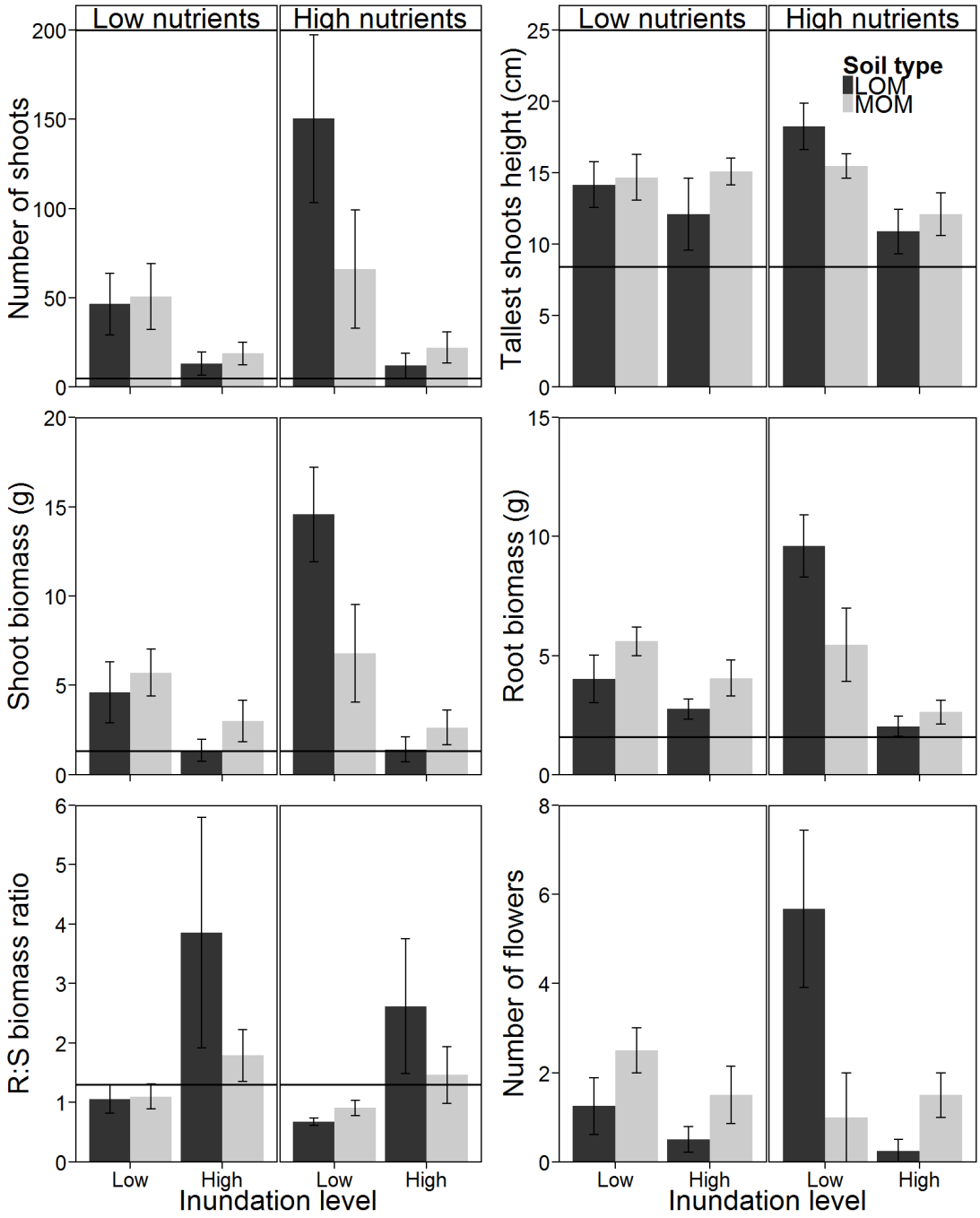

Figure 2

Number of shoots, shoot height, shoot biomass, root biomass, R:S biomass ratio and number of flowers of *Spartina* from Bellocchio in response to different inundation levels (low vs. high), nutrient levels (low vs. high), and soil type (low organic matter, LOM vs. medium organic matter, MOM). Horizontal lines indicate baseline values measured from the reference *Spartina* at the start of experiment. Data are average amounts per pot (n= 3 - 4, ± 1 SE).

Table 3 Number of pots with alive or dead plants in October 2013 and number of missing pots due to a storm just after the set-up. Percent survival was calculated as number of pots with alive plants divided by number of available pots at start (four replicates minus missing pots).

Plant source	Nutrient	Inundation level	Soil	No. alive	No. dead	No. missing	Survival (%)
Bellocchio	Low	Low	LOM	4	-	-	100
			MOM	4	-	-	100
		High	LOM	4	-	-	100
			MOM	4	-	-	100
	High	Low	LOM	3	-	1	100
			MOM	4	-	-	100
		High	LOM	4	-	-	100
			MOM	4	-	-	100
Vallona	Low	Low	LOM	4	-	-	100
			MOM	3	1	-	75
		High	LOM	-	3	1	0
			MOM	2	1	1	67
	High	Low	LOM	3	1	-	75
			MOM	4	-	-	100
		High	LOM	2	2	-	50
			MOM	2	1	1	67

Table 4 PERMANOVA analysis of the effects of inundation level (In, low vs. high), nutrient level (Nu, low vs. high) and soil type (So, LOM vs. MOM) on nine response variables for *Spartina* plants sourced from Bellocchio. Here the simplified model is shown, where highly non-significant interactions terms (p>0.25) were sequentially removed following Underwood (1997) and Anderson *et al.* (2008) (see Appendix S2 for the full models). Significant p values (p<0.05) are in bold, while marginally significant values (0.05<p<0.1) are in italics.

	No. of shoots				**No. of flowers**			**Shoot biomass**		
Source	**df**	**MS**	**F**	**P**	**MS**	**F**	**P**	**MS**	**F**	**P**
In	1	29482	18.55	0.0001	21.33	10.19	0.004	260.4	26.67	0.0001
Nu	1	7067	4.45	0.046	3.41	1.63	0.221	55.4	5.67	0.023
So	1	1987	1.25	0.281	0.65	0.31	0.591	6.9	0.71	0.411
In x Nu	1	6552	4.12	0.049	4.81	2.3	0.149	62.3	6.38	0.016
In x So	1	4408	2.77	0.114	15.41	7.36	0.012	43.7	4.48	0.047
Nu x So	1	3414	2.15	0.157	15.41	7.36	0.013	41.4	4.24	0.051
In x Nu x So	1	4137	2.6	0.125	18.25	8.72	0.007	34.2	3.5	0.071
Residual	23	1589			2.09			9.8		
Total	30									

	Root biomass				**Mean tallest shoot height**			**Root: shoot biomass ratio**		
Source	**df**	**MS**	**F**	**P**	**MS**	**F**	**P**	**MS**	**F**	**P**
In	1	84	27.86	0.0002	70.8	7.33	0.011	17.4	6.61	0.006
Nu	1	5.1	1.71	0.21	0.1	0.01	0.922	2.3	0.86	0.4
So	1	0.2	0.07	0.792	2.3	0.23	0.633	4.1	1.56	0.239
In x Nu	1	27.5	9.14	0.006	37.7	3.91	0.058			
In x So	1	9.6	3.19	0.085	18.4	1.9	0.181	5.9	2.26	0.15
Nu x So	1	19.8	6.58	0.015						
In x Nu x So	1	12.1	4.02	0.058						
Residual	23	3								
Pooled	25*				9.7			2.6		
Total	30									

	Soil loss				Soil redox		
Source	df	MS	F	P	MS	F	P
In	1	0. 79808	1.5076	0.2308	0.0028525	1.3091	0.2621
Nu	1	0.72242	1.3647	0.2583	0.0018238	0.83698	0.371
So	1	0.33743	0.63742	0.4441	0.005326	2.4442	0.1261
In x So	1	3.1992	6.0434	0.0224			
Pooled	26**	0.53652					
Total	30						

*25 for mean tallest shoot height and 26 for root:shoot biomass

**26 for soil loss and 27 for soil redox

A3.4 Discussion

The simulated inundation increase had a strong negative effect on most *Spartina* plant growth parameters, particularly in LOM soils. The R:S biomass ratio increase in the higher inundation treatments reflected a greater tolerance of roots than shoots to increased inundation, rather than an increase in root biomass. These negative effects of increased inundation on *Spartina* biomass production corroborated with results of other mesocosm experiments (Voss, Christian & Morris 2012; Watson *et al.* 2014). However, our plants were more sensitive than reported in experiments with the same species from other regions (Adams & Bate (1995) in South Africa; Castillo *et al.* (2000) in Odiel, Spain), as also suggested by a recent large-scale, marsh-organ experiment replicated at several locations around Europe including the north Adriatic sea (van Belzen *et al.* unpublished data). This distinct negative response might be related to the microtidal nature of the Mediterranean system where *Spartina* grows in a relatively narrow range of elevations (Ibáñez *et al.* 2000), such that it may be less adapted to large sustained changes in inundation. It could also indicate that the current elevation of the *Spartina* is already at or below the elevation for optimal growth; theoretical models and empirical data predict that saltmarshes at elevations below the optimum level are more vulnerable to increasing relative sea level compared to systems that are located at higher than optimal elevations (Morris *et al.* 2002; Kirwan & Guntenspergen 2012).

Responses to treatments differed between plants from different source locations; increased inundation resulted in particularly high mortality for plants sourced from Vallona Lagoon. We exclude that this particularly negative response was related to any experimental procedure, as it was not observed for plants from Bellocchio, which were subjected to the greatest potential stress due to transportation to the Vallona Lagoon study site. The surviving plants from Vallona had lower growth rates, biomass and reproductive potential than plants from Bellocchio, and did not seem to respond to the nutrient addition. One possible hypothesis is that the Vallona strain might be a distinct cryptic species previously undescribed in our study region. Preliminary results from a broad-scale genetic study, conducted one year after the conclusion of this experiment and still in progress, indicate the presence of the alien *S. anglica* in our study region, including the Vallona lagoon (Wong *et al.*, unpublished data). This invasive hybrid has hitherto been un-documented in this region likely due to difficulties in identification via morphology and similarities in habitat with the native *S. maritima*. We are now trying to clarify the exact distribution and abundance of the invasive species in

our region using molecular tools. Whether the two strains are different cryptic species or discrete phenotypes, this result suggests that site-specific responses to increased inundation at the foreshore of saltmarshes can be extremely variable, and should be incorporated into regional conservation planning.

The effects of increased inundation on *Spartina* were modified by nutrient conditions and soil type. Our hypothesis that low nutrient conditions would mitigate the negative effects of increased inundation by maintaining greater R:S ratio was only partially supported by our results. On average, biomass differences between inundation treatments were stronger at high than low nutrient conditions, particularly in LOM soils. This suggests that saltmarshes in high nutrient environments will face larger decreases in biomass with increased inundation as a result of accelerated sea level rise than saltmarshes in low nutrient environments, and that this will be particularly relevant for saltmarshes in LOM soils that are often associated with younger saltmarshes. However, the expected benefits of low nutrients on root biomass in plants experiencing higher inundation were less than predicted, and did not result in significant improvements in root growth. We hypothesize that the distinct large negative effects of increased inundation may have masked nutrient effects. A study that manipulated inundation, nutrients and CO_2 in tandem (Langley *et al.* 2013) also found that inundation was the dominant stressor on biomass production and the R:S ratio, although nutrient addition increased growth and conferred greater survival to saltmarsh plants under moderately increased flooding conditions. In contrast, another mesocosm experiment manipulating inundation, nutrient availability and precipitation with *S. alterniflora L.* found no effect of nutrient increase on root and shoot biomass, although root morphology shifted from coarse to finer roots with increased nutrients (Watson *et al.* 2014). The variability of saltmarsh root growth to increased nutrients suggests that responses are likely to be case specific, dependent upon soil type, species and magnitude of enrichment (see also Table 1).

Smaller biomass differences between nutrient treatments in MOM soil (compared to LOM soil) support our second hypothesis that MOM soils can dampen the effect of increased nutrients on root growth. MOM soil is more efficient in removing additional inorganic nitrogen species compared to LOM soil via a combination of denitrification and the coupled nitrification-denitrification of ammonia. Under anaerobic soil conditions, denitrifying bacterial activities result in the removal of nitrates and consumption of organic carbon with production of dissolved organic carbon (Ingersoll & Baker 1998). In coupled nitrification-denitrification, ammonia is first converted to nitrite then to nitrate by nitrifying bacteria followed by denitrification in a coupled process (An &

Joye 2001). Coupled nitrification-denitrification is likely to be the predominant pathway for nitrogen loss in saltmarsh platforms (Koop-Jakobsen & Giblin 2010) although other biotic and abiotic pathways also play a role (Burgin & Hamilton 2007), such as the physical flushing of nutrients from the soil porewater. The latter mechanism tends to be more important in well-drained sandy substrates than in cohesive organic-rich substrates (Thompson *et al.* 1995). Thus, although organic soils supporting mature saltmarshes have large amounts of nitrogen locked up in the soil particulate organic matter compared to young saltmarshes (Craft *et al.* 1988; Langis, Zalejko & Zedler 1991), they also support high rates of denitrification resulting from low oxygen levels and the longer retention time of dissolved nutrients in soil porewater (Thompson *et al.* 1995).

Measuring differences in soil erosion as a consequence of inundation and nutrient concentrations in the environment was beyond the scope of this manuscript, and it would have required specific dedicated experiments using undisturbed soil cores. Nevertheless, the largest loss of soil observed in pots with MOM soils under increased inundation is consistent with results from other work suggesting that large organic detritus reduces soil cohesiveness (Feagin *et al.* 2009). Additionally, a high nutrient availability can increase rates of paired sulfate reduction and organic matter decomposition (Watson *et al.* 2014) which could have further reduced soil volume, leading to soil elevation loss within the pots. These preliminary observations suggest that future work would be worth to further explore the links between nutrient concentrations, vegetation growth and soil erodibility, and how these could be modified under different inundation regimes.

A3.4.5 Conclusion and implications for management

Complex interactions between nutrients and various natural and anthropogenic factors are known from many marine ecosystems (e.g. Breitburg *et al.* 2009; Falkenberg *et al.* 2013; Strain *et al.* 2014; Thrush *et al.* 2014). This first work on the effect of nutrient levels and soil type in modifying the effect of increased inundation on saltmarsh vegetation reveals empirical evidence that can assist in the implementation of ecosystem-based management for salt marshes under accelerated sea level rise. Even though increasing inundation as a result of accelerated sea level rise will be gradual and likely result in longer term vegetation growth and sedimentation feedbacks, our results provide an indication of the expected direction of change under the different simulated scenarios.

Overall, our results supported the hypothesis that increased inundation would have a negative effect on *Spartina* survival and biomass production and that the reduction in productivity would be greatest in LOM soils representing young saltmarshes, particularly at high nutrient levels.

Newly created saltmarshes may therefore be effective nitrogen sinks to mediate excess nutrients arising from eutrophication; however, this service will be severely hampered if the saltmarsh elevation is unable to keep up vertically with sea level rise, or if saltmarsh zones are unable to migrate to higher elevations, e.g. due to presence of hard structures (Donnelly & Bertness 2001) or lack of upland marshes as in saltmarsh islands (Schile *et al.* 2014). Finally, the effect of nutrient enrichment (and conversely nutrient management) on *Spartina* vegetation is likely to be very site and condition specific. Managers should therefore be careful not to generalize effects between different saltmarshes.

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Supporting Information

A3.6 Appendices

Appendix S1- Selection of treatment nitrate concentrations

We simulated the "high" nutrient scenario based on the nitrate concentrations of Caorle Lagoon. This lagoon frequently has the highest nitrate concentrations among all the major lagoons in the Veneto region. Based on data collected by ARPAV from 2009 to 2012 (ARPAV 2011, 2012, 2013a, 2013b), we set the target "high" concentration to be 1.5mg/L of nitrate-N which is reflective of the average median nitrate concentrations for Caorle Lagoon (Figure S1.1).

We conducted pre-tests with different fertilizer doses of slow-release fertilizer Osmocote ® Exact Standard NPK in un-vegetated pots in the Vallona study site. The N component of this fertilizer is comprised of nitrate nitrogen (NO_3-N) (6.6 %) and ammonium nitrogen (NH_4-N) (8.4 %) (data from www.everris.com). The fertilizer dose of 45g (the maximum dose tested) was deemed sufficient to achieve the target "high" nitrate concentration 1.5mg/L in pore water (Figure S1.2).

Nutrient treatment had an effect on *Spartina* from Bellocchio, but not from Vallona. Compared to low nutrient conditions, high nutrient conditions resulted in higher percentage nitrogen and lower C: N ratios in roots from the Bellocchio plants (Figure S1.3), but the same trend was not detected in Vallona plants (Figure S1.4).

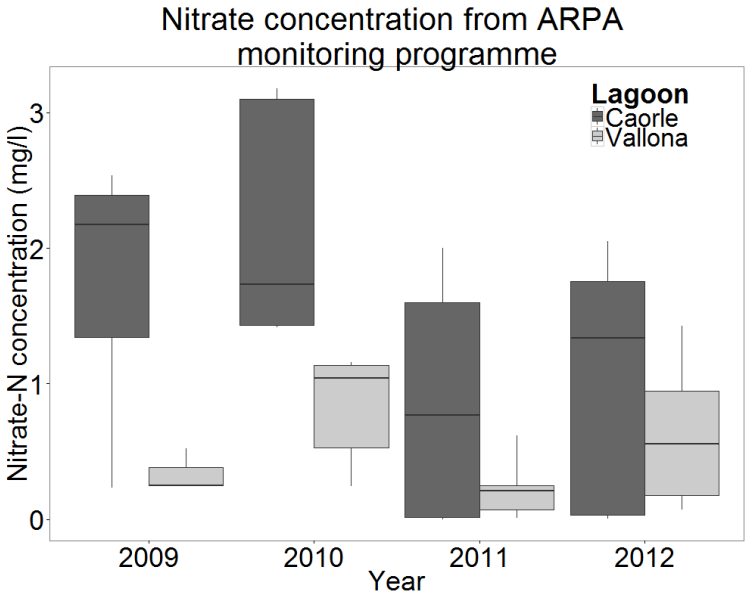

Figure S1.1 Boxplot of nitrate-N concentrations measured in Caorle and Vallona in the Veneto region from 2009 to 2012 show consistently higher nitrate levels in the Caorle Lagoon (based on ARPAV, 2011, 2012, 2013a, 2013b).

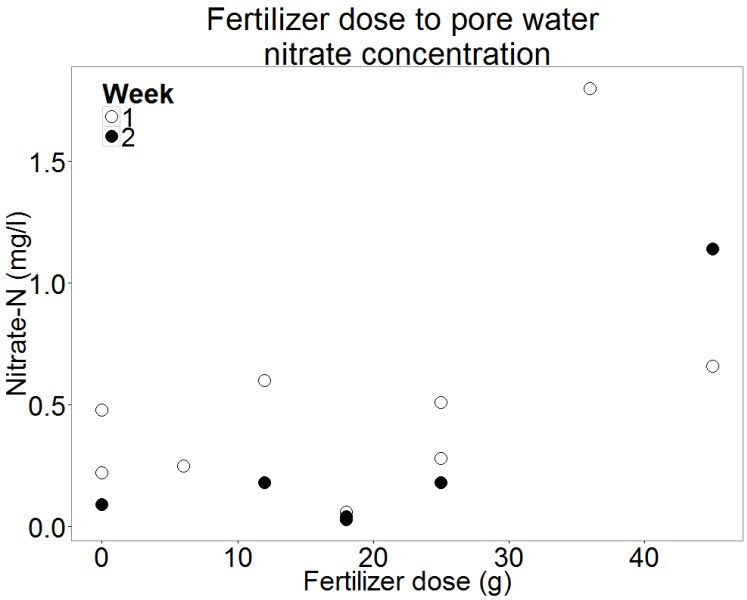

Figure S1.2 Nitrate-N concentrations of pore water measured one and two weeks after dosing with Osmocote ® Exact Standard NPK fertilizer in a pilot test.

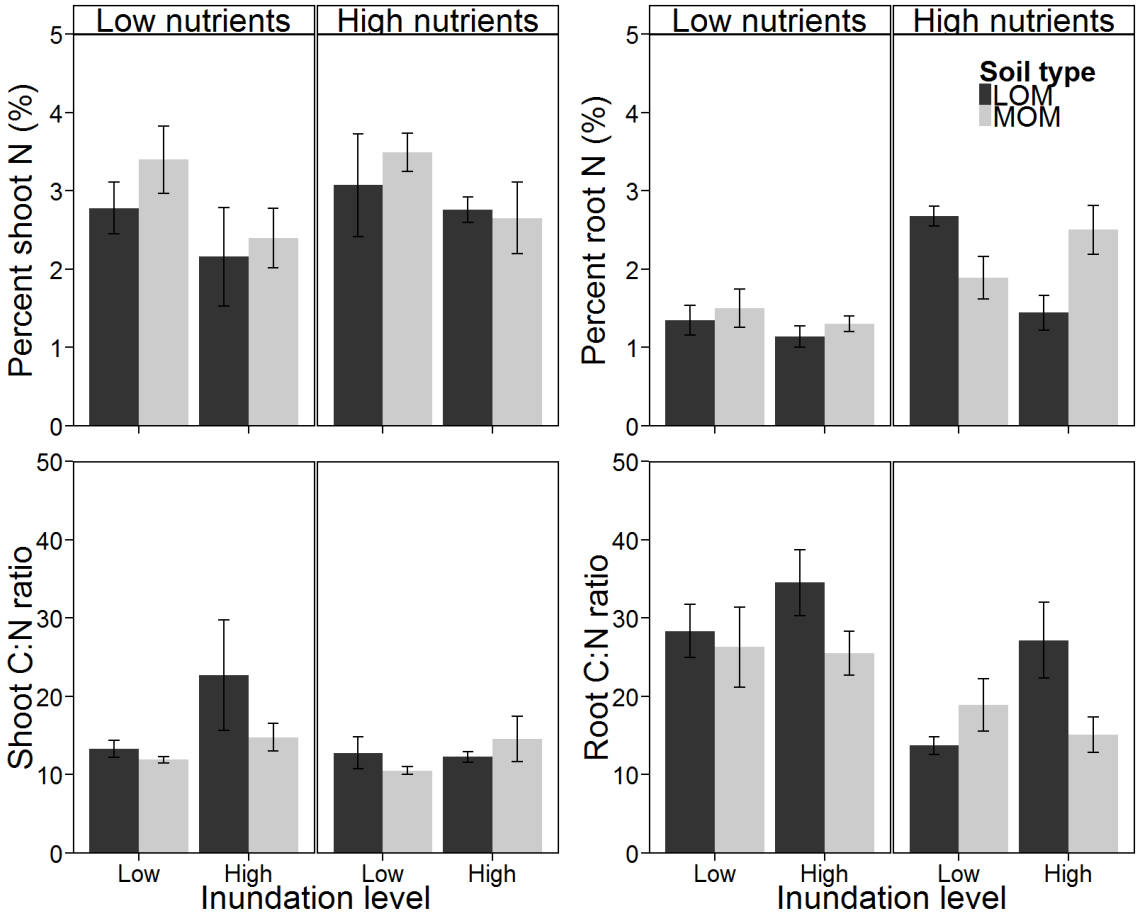

Figure S1.3

Root and shoot percent nitrogen and C:N ratio of *Spartina* from Bellocchio in response to different inundation level (low vs. high), nutrient levels (low vs. high), and soil type (LOM vs MOM). Data are average amounts per pot (n= 3 - 4, ± 1 SE).

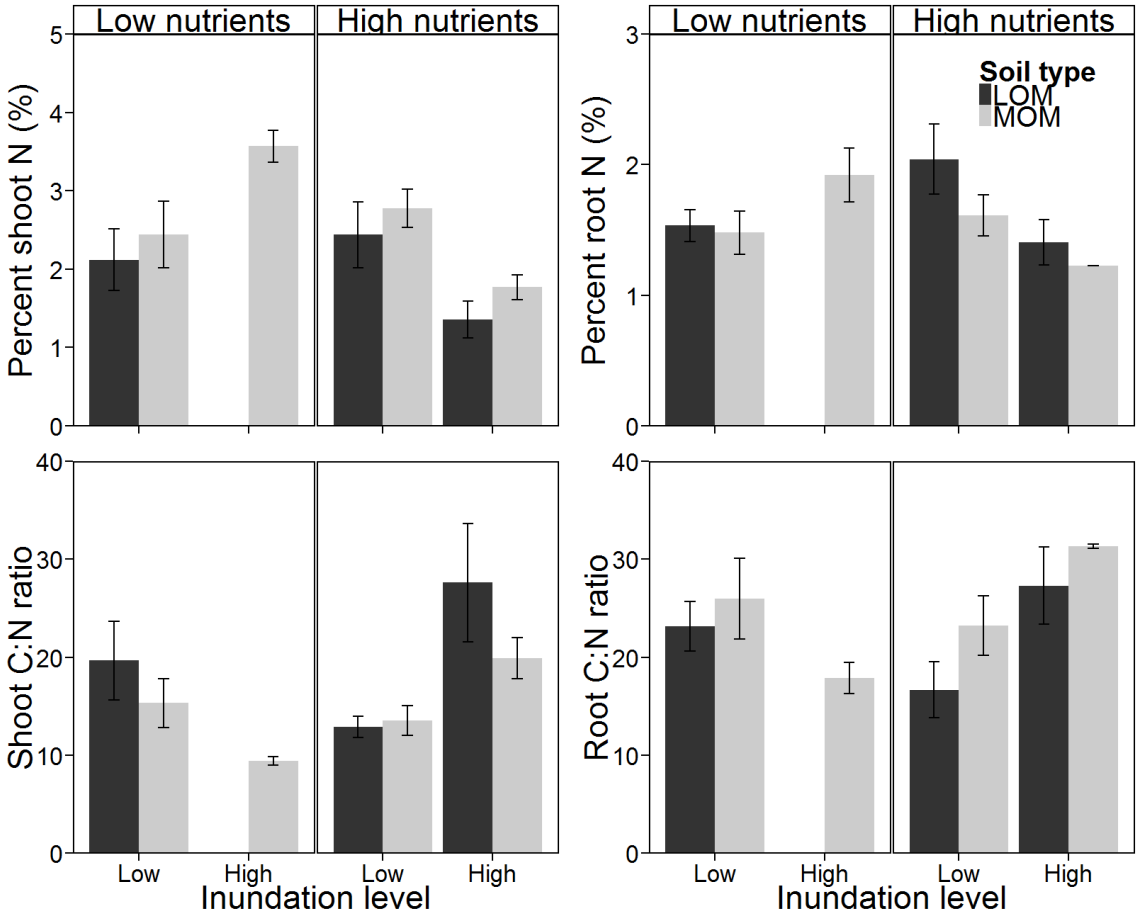

Figure S1.4 Root and shoot percent nitrogen and C:N ratio of *Spartina* from Vallona in response to different inundation level (low vs. high), nutrient levels (low vs. high), and soil type (LOM vs MOM). Data are average amounts per pot (n= 2 - 4, ± 1 SE).

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Appendix S2- Full PERMANOVA model results and figures

Table S2 PERMANOVA analysis of the effects of sea level (In, low vs high), nutrient level (Nu, low vs high concentrations) and soil type (So, LOM vs MOM) on 8 response variables for *Spartina* plants sourced from Bellocchio. Here the full models are shown.

	No. of shoots				**No. of flowers**				**Shoot biomass**			
Source	**df**	**MS**	**F**	**P**	**df**	**MS**	**F**	**P**	**df**	**MS**	**F**	**P**
In	1	29482	18.55	0.0001	1	21.333	10.187	0.0036	1	260.4	26.666	0.0001
Nu	1	7066.5	4.4461	0.0457	1	3.4133	1.6299	0.2214	1	55.384	5.6715	0.0233
So	1	1986.6	1.2499	0.2807	1	0.65333	0.31197	0.5911	1	6.9008	0.70666	0.4105
In x Nu	1	6552	4.1224	0.0488	1	4.8133	2.2984	0.1486	1	62.29	6.3786	0.016
In x So	1	4408.3	2.7737	0.1137	1	15.413	7.36	0.0117	1	43.701	4.4751	0.0473
In x So	1	3413.8	2.1479	0.1568	1	15.413	7.36	0.013	1	41.441	4.2436	0.0514
In x Nu x So	1	4136.7	2.6027	0.1251	1	18.253	8.7161	0.0074	1	34.206	3.5027	0.0707
Residual	23	1589.4			23	2.0942			23	9.7654		
Total	30				30				30			
	Root biomass				**Mean tallest shoot height**				**Root: shoot biomass ratio**			
Source	**df**	**MS**	**F**	**P**	**df**	**MS**	**F**	**P**	**df**	**MS**	**F**	**P**
In	1	83.952	27.858	0.0002	1	73.616	7.4202	0.0129	1	17.144	5.895	0.0147
Nu	1	5.1483	1.7084	0.2102	1	0.24539	2.47E-02	0.8747	1	2.1951	0.75478	0.4226
So	1	0.2187	7.26E-02	0.7919	1	1.748	0.17619	0.6733	1	4.1517	1.4276	0.2567
In x Nu	1	27.543	9.1395	0.0063	1	39.822	4.0138	0.0582	1	0.469	0.16127	0.7125
In x So	1	9.6123	3.1897	0.0845	1	19.856	2.0014	0.1732	1	5.8319	2.0053	0.1732
Nu x So	1	19.815	6.5751	0.0153	1	1.24E+01	1.2522	0.2759	1	0.57676	0.19832	0.6858
In x Nu x So	1	12.12	4.0219	0.0578	1	1.1322	0.11412	0.7378	1	0.24798	8.53E-02	0.7911
Residual	23	3.0136			23	9.9211			23	2.9082		
Total	30				30				30			
	Soil loss				**Soil redox**			
Source	**df**	**MS**	**F**	**P**	**df**	**MS**	**F**	**P**
In	1	0.78797	1.3549	0.2508	1	2.87E-03	1.198	0.291
Nu	1	0.71297	1.2259	0.284	1	1.78E-03	0.74416	0.389
So	1	0.33167	0.5703	0.4755	1	5.35E-03	2.2291	0.1515
In x Nu	1	4.22E-03	7.25E-03	0.9352	1	1.86E-03	0.77429	0.3907
In x So	1	3.1982	5.4992	0.0297	1	2.62E-04	0.10924	0.7434
Nu x So	1	0.19892	0.34204	0.5722	1	1.18E-03	0.49322	0.4861
In x Nu x So	1	0.16922	0.29097	0.5995	1	2.35E-04	9.81E-02	0.7645
Residual	23	0.58157			23	2.40E-03		
Total	30				30			

Appendix S3- Vallona result graphs

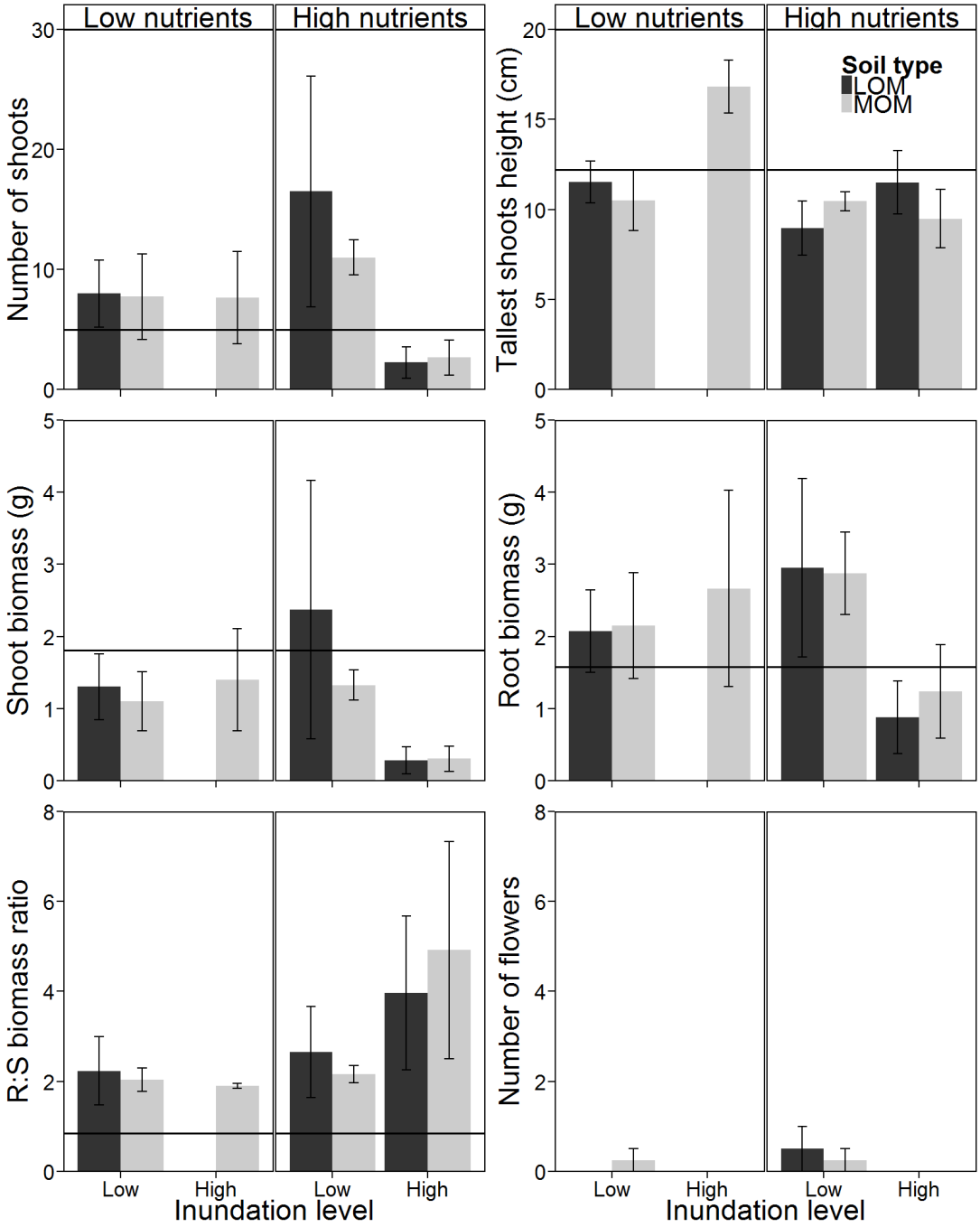

Figure S3 Number of shoots, shoot height, shoot biomass, root biomass, R:S biomass ratio and soil surface elevation loss of *Spartina* from Vallona in response to different sea levels (current vs future), nutrient levels (low vs high), and soil type (mineral vs organic). Horizontal lines indicate baseline values measured from the reference *Spartina* at the start of experiment. Data are average amounts per pot (n= 2 - 4, ± 1 SE).

Article 4 Effects of prevention of co-occurring and interacting local stressors in depleted *Spartina* habitats

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Abstract

Cumulative and interactive consequences of multiple human stressors are largely ignored in management plans. Further, these relationships are mostly known from studies focusing on increasing human pressures, while the reversal effects of remediation are largely unexplored. Experiments simulating realistic management scenarios of co-occurring and potentially interacting local stressors are crucial to understand the feasibility and cost-effectiveness of interventions geared towards recovery. We addressed how depleted ecosystems respond to simulated management scenarios of co-occurring anthropogenic local stressors that were reduced either individually or in combination. We orthogonally prevented two local stressors in depleted *Spartina* low intertidal habitats along the highly urbanized North Adriatic coastline (Italy), specifically damage from recreational trampling and smothering by deposition of excess macroalgal wrack due to high nutrient loads. Trampling and wrack prevention had interacting effects, causing non-linear responses of the vegetation to simulated management of these stressors. During the first year, *Spartina* shoot density increased only in those treatments where both stressors were prevented. When either trampling or wrack smothering was removed individually, no significant benefits were observed. During the second year, the benefits of the combined prevention of both stressors became less evident, while prevention of trampling individually caused adverse effects. The results demonstrate the challenges posed by synergisms between management actions, as the consequences of

an impact-by-impact approach could be ineffective if not negative. A clear understanding of the potential interactive consequences of remediation interventions is crucial to promote a more holistic management of multiple stressors in the coastal zone and achieve effective remediation of damaged ecosystems.

A4.1 Introduction

Most natural systems are affected by multiple human stressors (Halpern *et al.* 2008), which can interact causing unexpected non-additive cumulative effects (Crain, Kroeker & Halpern 2008; Darling & Côté 2008; Brown *et al.* 2013). However, current management of cumulative stressors occurs in a piece-wise manner (Halpern *et al.* 2008). While it is recognized that stressor interactions abound, significant knowledge gaps exists in understanding where and when interactions occur, and how they could affect management outcomes (Strain *et al.* 2015). Effective management decisions rely on good understanding of interactions (Halpern *et al.* 2008). Where multiple stressors have additive effects their management could be relatively straightforward and consistent with traditional impact-by -impact approaches. Whenever stressors interact, however, their management becomes more challenging, demanding for knowledge-based prioritization of the interventions (Halpern & Fujita 2013; Brown *et al.* 2013; Falkenberg, Connell & Russell 2013).

Experimentation is an important tool for the identification of interactions and feedbacks that constrain or facilitate recovery (Suding, Gross & Houseman 2004). In marine systems, multiple stressor field experiments have mostly focused on quantifying the combined effect of increasing multiple human stressors. These experiments have shown that interactions between multiple stressors can be common and concerning (Crain *et al.* 2008); for example, we know now that the negative effects of nutrient enrichment on benthic species can be amplified by the lack of herbivores and canopy forming algae (Russell & Connell 2005) and that massive saltmarsh dieback can be triggered by the combined effects of drought and over-fishing leading to cascading outbreaks of rampant snail herbivory (Silliman & Bertness 2002; Silliman *et al.* 2005). Although reduction of human impacts is the main driver for recovery (Lotze *et al.* 2006), the reversal effects of remediation of multiple stressors are still largely unexplored experimentally, likely because of greater difficulties in reducing than increasing most stressors particularly in the field (Strain *et al.* 2015). This knowledge is crucial as the trajectory for recovery may not mirror the pathway of degradation from stressor increase due to hysteresis as a result of altered competitive or trophic interactions (Halpern *et al.* 2008; Ling *et al.* 2009; Perkol-Finkel & Airoldi

2010). For example, field experiments have shown that overfishing and nutrient enrichment jointly affect coral reef ecosystems shifting them into a stable, algal-dominated state (Jompa & McCook 2002; McClanahan *et al.* 2003); however, this knowledge, while relevant, does not directly advice managers about how and to what level to address this interaction to reverse the system as there could be levels of fisheries removals and nutrient loadings that are resistant to remediation through fisheries or nutrient management alone. Research is urgently needed to increase our understanding of reversing stressor interactions, and the feasibility and cost-effectiveness of restoration in a globally changing world (Breitburg *et al.* 2009; Russell *et al.* 2009; Brown *et al.* 2013)

Estuaries worldwide offer many examples of systems under multiple human pressures due to their historic importance as transport links and eventual development into urban hubs (Valiela *et al.* 2009). Saltmarshes, which often occur in estuaries, have experienced large reductions in extent as a result of human modification and remaining saltmarshes continue to be under strong anthropogenic influence as a result of watershed pollution, land conversion, aquaculture and shipping (Gedan, Silliman & Bertness 2009). Given the economic and ecological importance of saltmarshes, and their vulnerability to further degradation from the presence of multiple stressors and ongoing climatic changes (Deegan *et al.* 2007; Ross & Adam 2013; Wong, Van Colen & Airoldi 2015) , they offer an ideal model system to investigate experimentally the potential effects of different management scenarios for co-occurring stressors.

Intensive trampling by recreational visitors and deposition of macroalgal wrack blooms (henceforth wrack) as a consequence of high nutrient loads are two local and experimentally manipulable pressures that occur in some saltmarsh systems. Trampling has been shown to reduce plant cover, height, biomass and species diversity in a number of grass-dominated systems, including saltmarshes (e.g. Eckrich & Holmquist, 2000; Goldman Martone & Wasson, 2008; Sun & Liddle, 1993; Talora, Magro, & Schilling, 2007), while macrophyte and macroalgal wrack cover in saltmarshes have been linked to reduced vegetation growth and cover as well as the spread of invasive grasses (Minchinton & Bertness 2003; Stalter *et al.* 2006; Hulzen *et al.* 2006). In a saltmarsh trampling study, Woolfolk (1999) observed that wrack tended to accumulate in experimentally trampled plots, indicating a potential interaction between these stressors.

The co-occurrence of these two stressors in space and time during the summer season provided an opportunity to examine the presence of potential interactions in combined management. To test how depleted saltmarsh vegetation along the north Adriatic coastal lagoons

(Italy) responds to simulated management scenarios of multiple anthropogenic local stressors, we conducted a two-year field experiment in which we (1) simulated realistic management scenarios where co-occurring trampling and wrack stressors were prevented singly or in tandem, and (2) measured the response in vegetative growth of the main perennial halophyte of the low intertidal in our study region, the cordgrass *Spartina maritima* (henceforth *Spartina*).

A4.2 Methods

A4.2.1 Study areas

The saltmarshes in the Italian Adriatic coast have a long history of human modification, degradation, and loss (Cencini 1998). The persisting saltmarshes are under continued threat, and face an uncertain future due to the combination of stressors at local (anthropogenic land subsidence, vessel wake erosion, hydrological modification, nutrient enrichment from land-based discharges, and tourism) and global (eustatic sea level rise, increased temperatures, changes in rainfall and salinity patterns) scales (Day 1998; Day *et al.* 1999; Silvestri, Defina & Marani 2005; Silvestri, Ghinoi & Barone 2013; Simeoni & Corbau 2009; Sarretta *et al.* 2010; Facca *et al.* 2011; Bellafiore *et al.* 2014; Villatoro *et al.* 2014). The research was conducted in two of the few remaining *Spartina* habitats in the region. Due to its perennial growth with dense above and belowground biomass this species is a relevant foundation species and plays an important role in substrate stabilization and erosion control (Neumeier & Ciavola 2004). The two *Spartina* habitats fringed the Sacca II Lagoon in the Bellocchio Nature Reserve (12.263421°, 44.630832°, henceforth Bellocchio) and the San Felice saltmarsh in Venice Lagoon (12.452916°, 45.485479°, henceforth Venice), along the north-east Italian coast. Both lagoons experience a semi-diurnal tide (Marani *et al.* 2006b) with a range of about 1 to 1.4 m. Bellocchio Lagoon is a small, back-barrier coastal lagoon (depth ~0.5m, area 6km^2) connected to the sea by a channel that is maintained by periodic dredging to maintain water quality (Villatoro *et al.* 2014). It is fed by a tributary of the river Reno. Venice Lagoon is a large shallow coastal lagoon (average depth ~1.1 m, area 550 km^2) (Belluco *et al.* 2006) connected to the Adriatic sea by three inlets and receiving freshwater inputs from a few tributaries, contributing a relatively small water flux but a relatively large associated input of solutes (e.g. nutrients from agricultural areas, Facca *et al.* 2011).

The salt marsh vegetation at both study sites is similar, showing distinct patterns of zonation (Silvestri *et al.* 2005) that are likely determined by a combination of elevation and other ecohydrological characteristics like soil oxygenation (Marani *et al.* 2006a). The surveys and experiments were conducted in the lower marsh which is primarily dominated by *Spartina*

tussocks (Silvestri *et al.* 2005; Merloni & Piccoli 2007) and interspersed with *Salicornia veneta* and *Sarcocornia fruticosa* from 0 to 30 cm above mean sea level (a.m.s.l.) (Strain *et al.* in preparation). All surveys and experiment were conducted during summer-autumn to coincide with the peak growth period for *Spartina*, as well as the highest wrack deposition (Hulzen *et al.* 2006) and coastal tourism pressure (Regione Emilia-Romagna 2014). The summer period brings peak numbers of visitors to Italy's beaches (Cuccia & Rizzo 2011), this human presence causes ecological impacts to coastal ecosystems particularly in areas close to beach access points (Tratalos *et al.* 2013). In principle access to the *Spartina* habitat is regulated, however due to limited enforcing we repeatedly observed people accessing them for a variety of activities (see results). Additionally, increasing surface water temperatures in summer lead to peak standing biomass of macroalgae (mainly *Ulva* spp) especially in eutrophic lagoons (Viaroli *et al.* 2005). The occurrence of these two disturbances coincides with the most active *Spartina* growth period, which can lead to particularly detrimental effects as carbohydrate production and storage are impeded (Hull, Sullivan & Lytle 1976).

A4.2.2 Quantification of wrack accumulation on *Spartina*

In June 2012 and July 2013, we carried out surveys to characterize the spatial and temporal cover of wrack on *Spartina* (Figure 1). The survey was done in Bellocchio lagoon only, due to permission restrictions in Venice lagoon. We quantified the percent cover of wrack and *Spartina* (absent: no *Spartina* shoots, healthy: green shoots present, degraded: brown shoots from the previous year with no green shoots) in the lower marsh using the intercepted-length method (Kingsford & C Battershill 1998). In both years, two 50 m line transects were randomly laid > 5 m apart and parallel to the waterfront, at 15 cm a.m.s.l. The lengths intersected along the transect for each of the 3 categories were recorded to the nearest cm, and the total length of each category type was expressed as a percentage of the total length. We used this data to identify whether there was a relationship between wrack cover and status of *Spartina*, by quantifying the percentage of healthy vs degraded *Spartina* covered or not covered by wrack. We only sampled along two transects as this length covered almost all the remnant *Spartina* tussocks along the accessible side of the lagoon.

Figure 1 Macroalgal wrack deposit over saltmarsh at the Bellocchio experimental site (top); top-down view of natura macroalgal wrack deposit over a *Spartina* PC plot.

We also set up eight 50cm x 50cm permanent quadrats in eight randomly selected *Spartina* tussocks in Bellocchio to record temporal changes in percent cover of wrack over the experimental period using photo quadrats. The permanent quadrats were surveyed 4 times each year (June to Oct in 2012 and July to October 2013). A metal quadrat frame was first laid over the *Spartina* tussocks and photo records of the quadrats were taken. The extent of *Spartina* vegetation covered by wrack in terms of percentage cover was visually estimated from photos to the nearest 5%.

A4.2.3 Quantification of trampling pressure on *Spartina*

Between June and October in 2012 and 2013, we recorded the incidence of human trampling in an area of approximately 0.7 ha of low intertidal with a mix of *Spartina* and *S. veneta* vegetation in Bellocchio. The number of people and duration of each trampling event was recorded; repeat tramplers from the same observation day (i.e. people who left the surveyed area but returned again at a later time) were counted as separate events. The surveys were carried out on five random days per year, where each survey day consisted of 4.5 hours of observations by one observer between 9am and 5pm when the site was open for public access.

A4.2.4 Field experiment

In June 2012, at each of the two study locations, Bellocchio and Venice, we set-up 16 experimental plots of 1m x 1m in individual *Spartina* tussocks of approximately 1-2m width. All plots had similar start-of-season shoot number (mean = 197, SE = ±7.3 for the central 50cm x 50cm of each plot). At each location, we randomly assigned 4 replicated plots to each of one orthogonal combination of two treatments simulating different intervention scenarios on the stressors: trampling by recreational visitors removed vs maintained; and deposition of wrack removed vs maintained (see Table 1 for details). Trampling prevention was achieved by fencing, which could represent a realistic management intervention (at larger spatial scales). Stress by wrack in these lagoons is a consequence of excess nutrient load, which was impossible to experimentally reduce. As an alternative we focused directly on the prevention of wrack accumulation, in order to simulate one of the benefits that could arise from a management control of nutrient loads in these lagoons. Each plot was fenced with four wooden corner posts and 1 cm mesh plastic net to exclude wrack and tramplers from entering the plots. In the treatments simulating the prevention of both wrack and trampling (hereafter W&T) we did not carry out further manipulations. In the treatments simulating the prevention of wrack only (hereafter W) ambient trampling stress was re-introduced by having an experimenter walk from one edge of the plot to the other edge and back again 10 times (for a total of 20 passes) per treatment. To avoid edge effects, the start, end and turning points of each pass fell outside the 1m x 1m plot (Cole & Bayfield 1993). In the treatments simulating the prevention of trampling only (hereafter T), ambient wrack deposition stress was reintroduced by spreading 2.9 kg of fresh *Ulva* spp. wrack evenly over the entire plot. We estimated the amount of fresh wrack required for each plot based on pre-test surveys of stranded wrack on the *Spartina* zone in Bellocchio in June and July 2012 (see Appendix). The wrack was collected from the adjacent lagoon, drip-dried for

15 seconds, and weighted in the field by using a portable balance. In the treatments simulating the ambient condition (hereafter = SimAmb) both stressors were reapplied as described previously. Treatments were applied twice (June and August) in 2012. In October 2012 we removed the fences to prevent damage from winter storms. This season also coincides with a period of low activity for *Spartina* (Ranwell 1967), macroalgal growth and tourist visits. The fences were re-constructed and the same treatments were re-applied in Bellocchio in July 2013 to capture multiple year treatment effects. The treatments were re-applied only once (end July) in 2013 because of the later start of blooms and lower amount of wrack in Bellocchio (see results). The experiment was not re-applied in Venice in 2013 due to difficulties in getting access permission.

In addition to the treatments simulating different intervention scenarios, we set-up four procedural control plots (hereafter PC) to identify potential effects of fencing in our experiment. These plots had corner posts but no fencing, and were subjected to similar levels of trampling as described in W treatments and to natural accumulation of wrack. In this way we tried to reproduce the same levels of stressors as in SimAmb treatments but without using the fencing. We also set up four ambient unmanipulated plots (hereafter Ambient) to estimate how well our SimAmb treatment simulated ambient conditions.

We measured shoot number, flower number and height of the five tallest shoots (hereafter referred to as shoot height) at the start-of-season (June or July) and end-of-season (October) each year. Measurements were taken in a 0.5m × 0.5 m quadrat laid in the center of each plot to exclude edge effects from the fencing. We measured the effect of the treatments on light penetration by measuring light availability 100 mm from the soil under the *Spartina* canopy using a Licor 250-A light meter at haphazard points (n= 8 – 10) in each plot immediately after the application of treatments. At the end of the experiment in Bellocchio (October 2013), we collected a core (100mm diameter and 150mm deep) from each plot to determine the effects of treatments on aboveground and belowground biomass of *Spartina*. We measured soil redox in each plot haphazardly (n= 2- 4) in Bellocchio at the conclusion of the experiment in 2013 with a handheld probe Hanna Instruments HI 8314 (with redox probe HI 3230).

Table 1 Overview of the experimental treatments (orthogonal combinations of wrack and trampling prevented, plus procedural control and unmanipulated ambient plots) and manipulations applied

Treatment	Manipulation
Both wrack and trampling prevented = W&T	Both trampling and accumulation of macroalgal wrack were excluded by using 1 cm mesh plastic net
Wrack prevented, trampling maintained = W	Accumulation of macroalgal wrack was excluded via plastic fencing. Because the fence also necessarily limits human access, trampling stress was re-applied to simulate ambient levels. This consisted in 20 passes per plot over the entire plot.
Trampling prevented, wrack maintained = T	Trampling was excluded via plastic fencing. Because the fence also necessarily limits wrack access, macroalgal wrack was re-applied to simulate ambient levels of accumulation. This consisted of 2.9kg of fresh macroalgae spread evenly over the entire plot.
Both wrack and trampling maintained (simulated ambient) = SimAmb	These treatments were fenced for consistency with the previous 3 treatments, but both trampling and macroalgal wrack were re-applied to simulate ambient levels for both stressors as described for W and T treatments.
Procedural control = PC	These treatments were included to identify potential artefact effects of fencing. We used unfenced plots, but subjected to the same known levels of trampling as described in SimAmb treatments. We tried to minimize extra ambient trampling by limiting access to them from land. Plots were subjected to natural accumulation of wrack.
Ambient levels of both stressors = Ambient	These treatments were unmanipulated plots exposed to natural ambient levels of both stressors.

A4.2.5 Data analysis

We modeled response variables using linear models (LMs) or generalized linear models (GLMs) as they offer more flexibility in analyzing non-normal data without requiring transformation of the response variable (Zuur *et al.* 2009). All statistical analyses were conducted using in R version 3.0.3 (R Core Team 2014).

We analysed the response variables for 2012 and 2013 separately. We first excluded the artefact effect of fencing on end-of-season *Spartina* shoot number by comparing the three levels of treatment (3 fixed levels= SimAmb, Ambient and PC). The analysis for 2012 also included site (2 fixed levels= Bellocchio and Venice). We employed a GLM with family negative binomial ("glm.nb" function in the MASS package) due to non-normality of residuals under an LM and over-dispersion under a Poisson distribution.

Response variables from 2012 were modelled individually against the three fixed factors: trampling (2 fixed levels = prevented and ambient), wrack deposition (2 fixed levels = prevented and ambient) and site (2 fixed levels= Bellocchio and Venice). Response variables from 2013 were modelled individually against two fixed factors: trampling (2 fixed levels = prevented and ambient) and wrack deposition (2 fixed levels = prevented and ambient). Shoot and flower number were modelled using GLMs with family negative binomial ("glm.nb" function in the MASS package) due to non-normality of residuals under an LM and over-dispersion under a Poisson distribution. Shoot height and biomass were modelled using LMs ("lm" function in the "lme4" package). For all response variables, we started with the most complex model with all main effects and two- and three-way interactions. Insignificant terms were removed by backward elimination one term at a time by calculating the chi-square. If the change was not significant ($p > 0.05$) the term was dropped from the final model. We checked for normality and homoscedasticity using qqplots and residual plots for LMs and using goodness of fit tests for GLMs.

Light availability under the *Spartina* canopy immediately after treatment application and soil redox at the end of the 2012 experiment were analyzed using the "gls" function from the nlme package (Pinheiro *et al.* 2014) using the compound symmetry auto-correlation structure argument, "corCompSymm", to account for similarity in variance from multiple readings per quadrat. Light measurements were square-root transformed to achieve normal distribution of residuals.

A4.3 Results

A4.3.6 Wrack survey

The average cover of wrack between transects was similar ranging from 79 to 82% (mean

= 80.8, SE = ± 1.0, n = 4). Wrack cover did not have a strong influence on *Spartina* condition as percent of healthy vs. degraded *Spartina* under wrack cover was highly variable between transects (Table 2).

Table 2 Total length along 50 m transects of wrack covering *Spartina* (healthy and degraded) and calculated percentage of total length over degraded and healthy *Spartina*.

	Transect 1	**Transect 2**	**Transect 3**	**Transect 4**
Total length of wrack over *Spartina*(healthy and degraded) (m)	4.9	27.6	23.1	4.2
% of total over degraded *Spartina*	81.6	47.5	35.5	19.0
% of total over healthy *Spartina*	18.4	52.5	64.5	81.0

The permanent photo quadrats (n= 8) surveyed 4 times each year in Bellocchio showed that in 2012 wrack accumulation on *Spartina* started in early July and peaked in October (mean = 56.3%, SE = ± 9.00) (Figure 2). In 2013, wrack accumulation started a month later in August but peaked earlier in September (mean = 27.5%, SE = ± 6.81) (Figure 2). Overall, peak cover in 2013 was lower than that in 2012. No wrack was observed in permanent quadrats in Venice during the experiment, however there was wrack covering the *Spartina* tussocks observed in other areas of the lagoon.

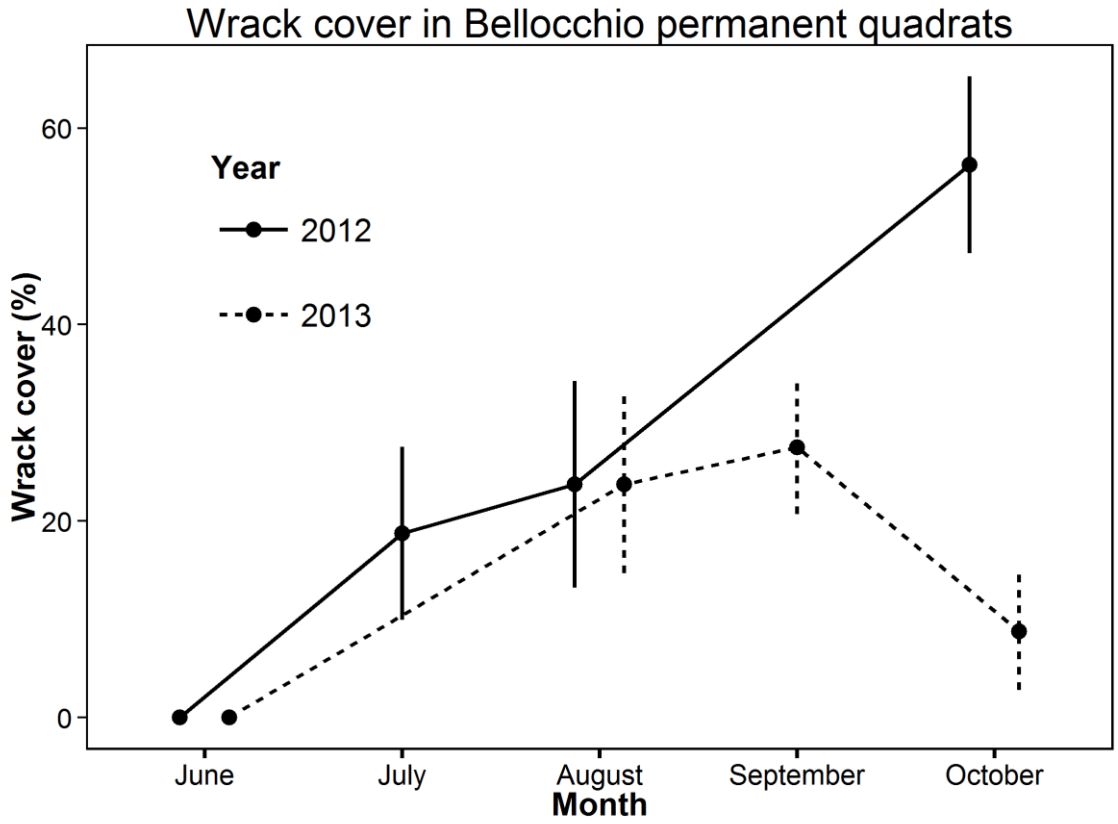

Figure 2 Monthly variability in the mean percent wrack cover (+/-SE) on *Spartina* in the 0.5 m × 0.5 m permanent photo quadrats in Bellocchio (n = 8) in 2012 and 2013.

A4.3.7 Trampling survey

Tramplers were observed walking on the intertidal saltmarsh zone in Bellocchio every day during the survey The number of tramplers per day of observation was highly variable, ranging from 2 to 17 (mean = 7, SE = ±1.8). The duration of each trampling event was also variable, ranging from 0.5 to 49 minutes (mean = 8.0, SE = ± 1.38). The tramplers were walking, taking photos or engaging in collecting activities within the intertidal area on the eastern side of the lagoon. We did not observe people entering the study site which is located on the western side of the saltmarsh lagoon during the surveys, but footprints that did not belong to the experimenters were observed during the course of the experiment.

A4.3.8 Field experiment

There were no detectable differences in shoot number between SimAmb, Ambient and PC (2012 shoot number: LR stat = 0.65, df = 2, p = 0.722; 2013 shoot number: LR stat = 0.68, p = 0.713). At the conclusion of the experiment in 2012, W&T had significantly higher number of *Spartina* shoots compared to the SimAmb treatment (GLM: z = 2.50, df = 1, p = 0.012; Table

3, Figure 3). In contrast, number of *Spartina* shoots in T (GLM: z = -1.29, df = 1, p = 0.197) and W (GLM: z = 0.31, df = 1, p = 0.753) was not significantly different to the SimAmb treatment (Table 3, Figure 3). Comparison of the shoot number final model with a reduced model without the trampling x wrack term showed that the interaction was significant (LR stat = 4.85, df = 1, p = 0.028; Figure 3). The number of *Spartina* shoots in the plots at Venice (mean = 66, SE = ±6.8) was significantly lower than in Bellocchio (mean = 235, SE = ±19.5) (GLM: z = -6.83, df = 1, p < 0.001) as many plants had flowered by October and had started to senesce, however the effects of the treatments were similar at both sites (Table 3, Figure 3). There was no detectable effect of treatment on the average height of the *Spartina* shoots (Table 3, Figure 4). Site was the only significant predictor for shoot height in 2012 where shoots in Venice were on average taller (mean = 47.7 cm, SE = ±1.623) than shoots in Bellocchio (mean = 34 cm, SE = ±3.115) (LM: t = 3.89, df = 1, p = 0.0005; Table 3). Wrack prevention resulted in an increase in the number of flowers (GLM: z =2.40, df = 1, p = 0.017; Table 3, Figure 5). There were 5x the number of flowers on the *Spartina* in Venice (mean = 40, SE = ±3.8) relative to Bellocchio (mean = 8, SE = ±1.7) (GLM: z = 4.83, df = 1, p = <0.0001; Figure 5).

At the end of the 2013 growing season, there were no detectable effects of treatments on the number of shoots (Figure 3), number of flowers (Figure 4), shoot height (Figure 5) and above- and belowground biomass (data not shown).

Following treatment application, light availability under the *Spartina* canopy was higher when wrack deposition was prevented than under ambient deposition (GLS: t = -2.96, df = 1, p = 0.004; Table 4 and Figure 6). There were no detectable effects of the treatments on soil redox (data not shown).

Table 3 Results of generalized linear model (GLM) and linear model (LM) testing the effects of trampling (2 fixed: prevented and ambient), wrack (2 fixed levels: 2 fixed: prevented and ambient) and their interactions on the number of shoots, number of flowers and mean height of shoots in Bellocchio and Venice 2012 (n=32). Tramp= trampling prevented, Wrack= wrack prevented, Site= Venice and z and t represents the z-test and t-test statistic. Significant P-values (<0.05) are shown in bold print.

Model and response variable	Source	df	z	P
GLM				
2012 Shoot number	Trampling	1	-1.29	0.1972
	Wrack	1	0.31	0.7538
	Site	1	-6.83	**<0.0001**
	Trampling : Wrack	1	2.50	**0.0123**
	Trampling : Site	1	1.40	0.1625
	Wrack : Site	1	-1.31	0.1920
	Residual	25	(deviance = 32.83)	
2012 Flower number	Trampling	1	-3.56	**0.0004**
	Wrack	1	2.40	**0.0166**
	Site	1	4.83	**<0.0001**
	Trampling : Site	1	4.77	**<0.0001**
	Residual	27	(deviance = 41.51)	
	Source	**df**	**t**	**P**
LM				
2012 Shoot height	Site	1	3.89	**0.0005**
	Residual	30	(S.E. = 9.936)	

Table 4 Results of generalized least squares (GLS) model testing the effects of trampling (2 fixed: prevented and ambient), wrack (2 fixed levels: 2 fixed: prevented and ambient) and their interactions on the light availability immediately after treatment application and soil redox at the end of the experiment in Bellocchio 2013. Multiple readings from the same quadrat were accounted for in the variance structure. Wrack= wrack prevented. Significant P-values (<0.05) are shown in bold print.

Model and response variable	Source	df	t	P
GLS model				
Square root (light availability)	Wrack	1	-2.96	**0.0038**
	Residual	116	(S.E. = 155.02)	

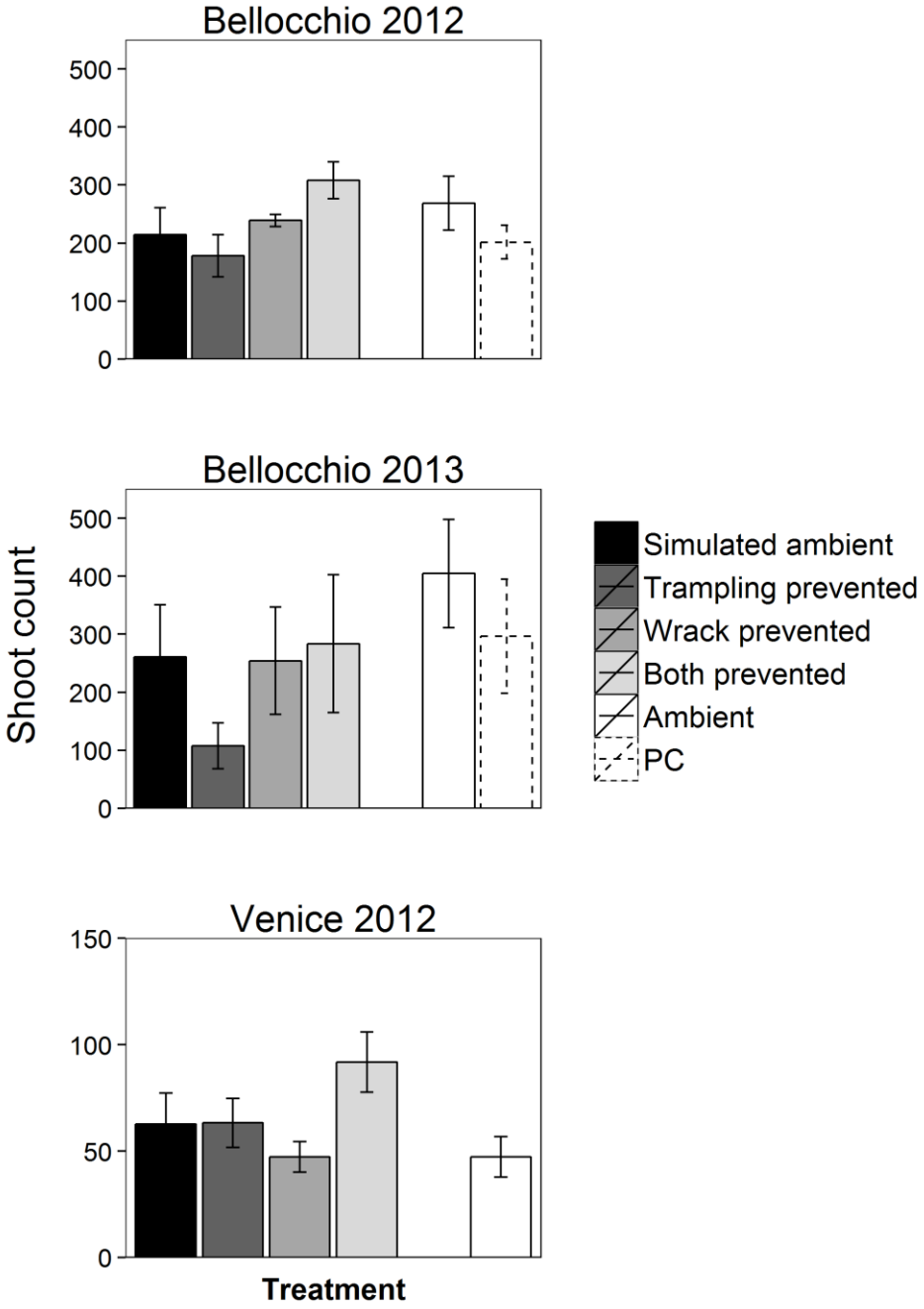

Figure 3 Effect of treatments on the mean *Spartina* shoot number (+/-SE) in Bellocchio (2012 and 2013) and Venice (2012 only) at the end of the growing season (October). Treatments are: simulated ambient, trampling prevented, wrack prevented, both stressors prevented, ambient and procedural control. Note the differing y-scales between Bellocchio and Venice.

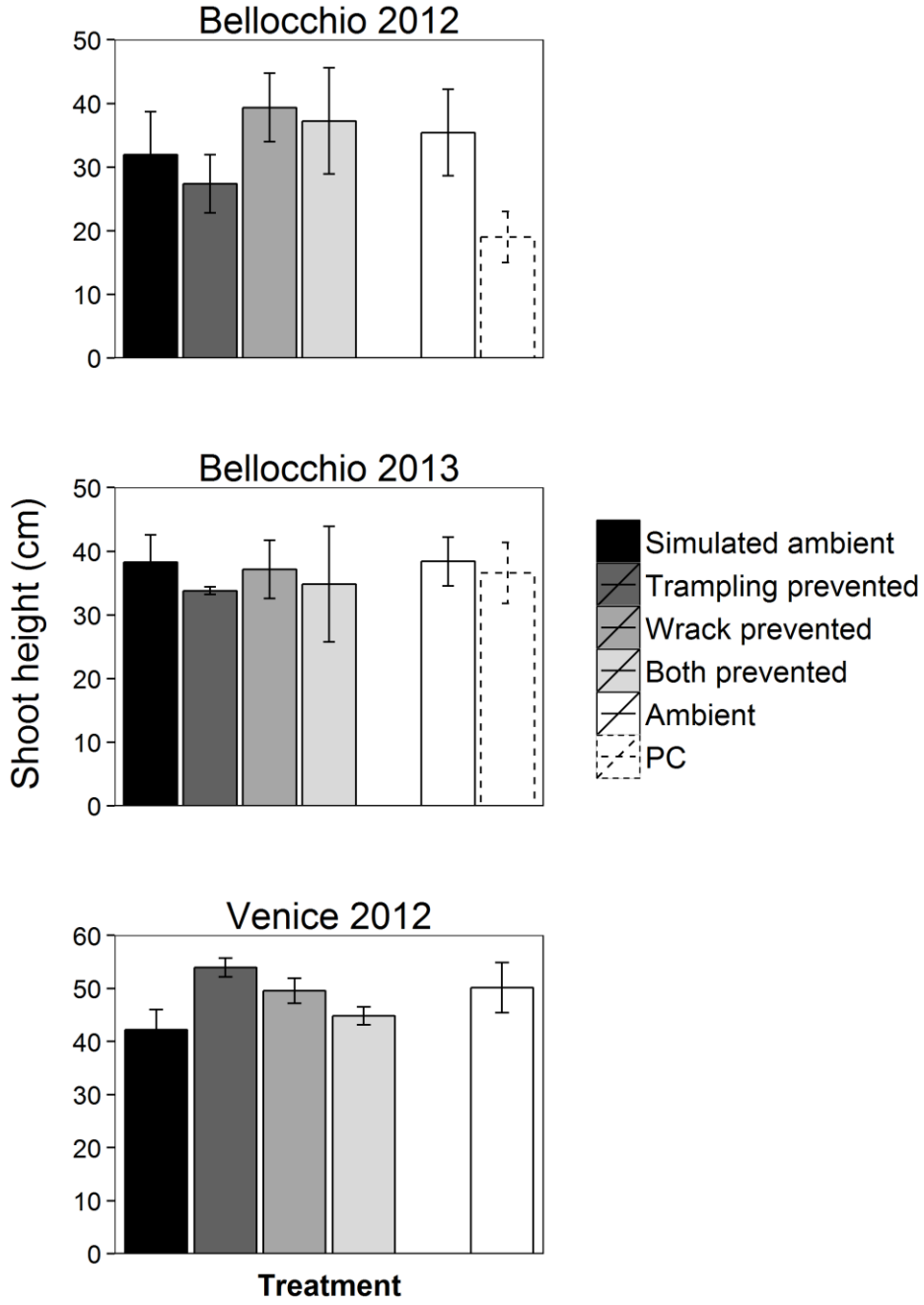

Figure 4 Effect of different management scenarios on the mean *Spartina* shoot height (+/-SE) in Bellocchio (2012 and 2013) and Venice (2012 only) at the end of the growing season (October). Treatments are: simulated ambient, trampling prevented, wrack prevented, both stressors prevented, ambient and procedural control. Note the differing y-scales between Bellocchio and Venice.

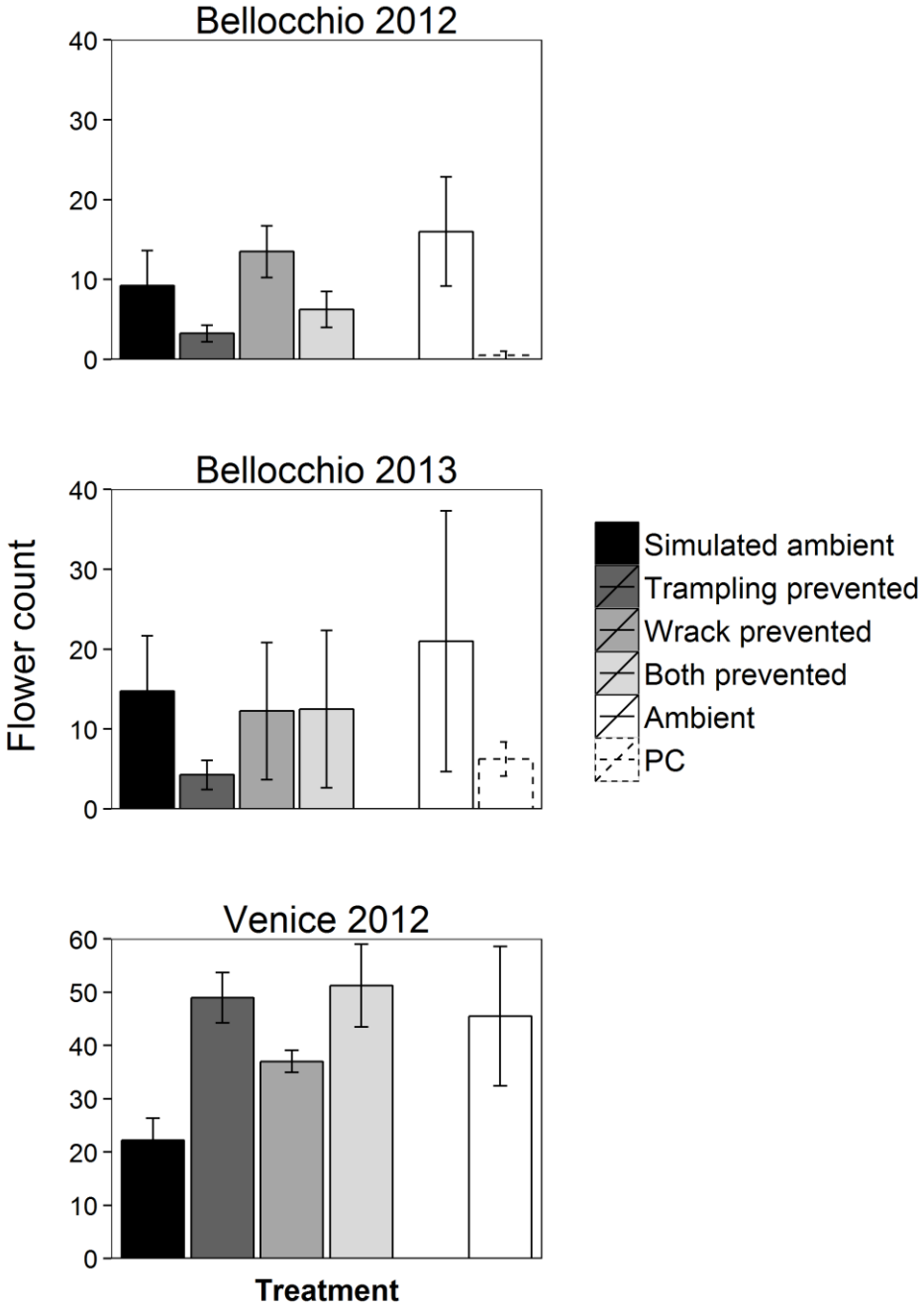

Figure 5 Effect of different management scenarios on the number of *Spartina* flowers (+/-SE) in Bellocchio (2012 and 2013) and Venice (2012 only) at the end of the growing season (October). Treatments are: simulated ambient, trampling prevented, wrack prevented, both stressors prevented, ambient and procedural control. Note the differing y-scales between Bellocchio and Venice.

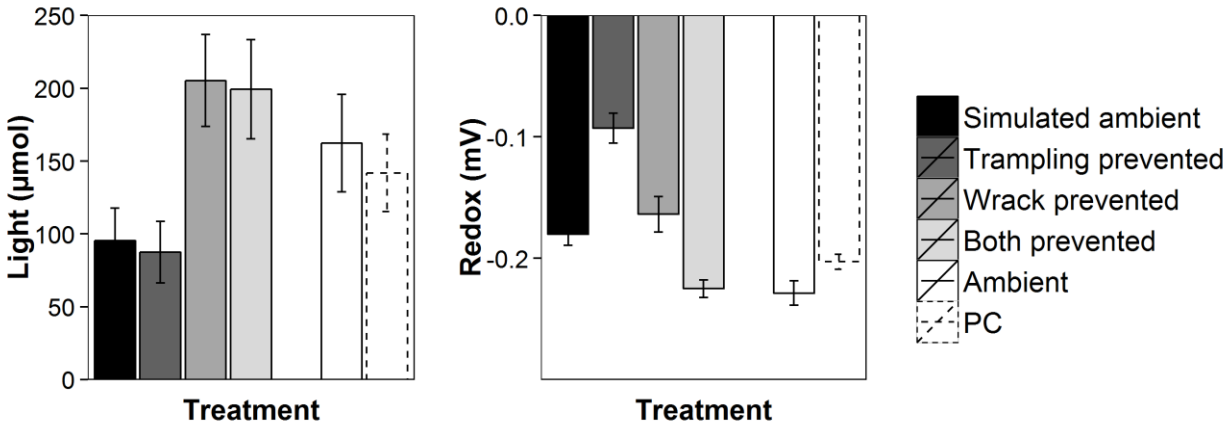

Figure 6 Effect of different management scenarios on the mean (+/- SE) light availability 10cm above the soil (left) and soil surface redox (right) under the *Spartina* canopy in Bellocchio after treatment application in 2013. Treatments are: simulated ambient, trampling prevented, wrack prevented, both stressors prevented, ambient and procedural control. Note the differing y-scales between Bellocchio and Venice.

A4.4 Discussion

The prevention of wrack deposition and trampling had a positive synergistic effect (after Piggott, Townsend & Matthaei 2015) on vegetation growth, implying that effective management in such cases may be more costly as multiple stressors need to be managed to achieve a beneficial response. While the exact mechanism could not be deduced from the measured responses, we speculate at two potential explanations: (1) each stressor individually could have a limiting effect on vegetation growth via different modes of action, such that the prevention of only one stressor had no significant beneficial effect on vegetation, or (2) the prevention of one stressor could have increased the sensitivity of the vegetation to the other, for example wrack prevention might have increased the negative effect of trampling by reducing the amount of detrital nutrient for regrowth, while preventing trampling might enhanced the negative light attenuation effects of wrack due to the even stem canopy cover.

There are few experimental studies that have tested the interaction effects between multiple management actions. One other study in saltmarshes detected significant interactions between the removal of two stressors: herbivory by invasive nutria and removal of nutrient limitation due to river channel restrictions (McFalls *et al.* 2010). The authors found that fertilization improved vegetation biomass only when nutria herbivory was also removed; herbivory was a strong limiting stressor such that fertilized plots without exclusion were not different from non-fertilized plots. Results from McFalls *et al.* (2010) and the current study

suggest that managing two local-scale stressors are not only feasible, but may also yield positive synergistic effects. Since trampling and macroalgal growth and subsequent deposition occur at a relatively local-scale, prevention of both stressors is likely to be feasible. For example, avoidance of trampling can be achieved by fencing, better signage and public education while wrack deposits could be reduced by mechanical harvesting in the short-term (Lenzi, Palmieri & Porrello 2003; M. *et al.* 2005) and improving the circulation of the Bellocchio Lagoon in the longer term (Leston, Lillebø & Pardal 2008).

Recreational trampling of the low intertidal saltmarsh (as well as the sand dune) was a regular occurrence in Bellocchio as tramplers were encountered during every survey day. The number of people accessing the saltmarsh area was observed to be higher closer to the car park and tourist facility on the beach. While we did not carry out trampling surveys in the San Felice saltmarsh in Venice Lagoon due to difficulties in access, it is likely that trampling pressure occurs here to some extent as it is not uncommon to see footprints in the saltmarsh intertidal zone. Trampling on grasses, even at a low level, can result a decrease in vegetation cover (Andersen 1995; Headley & Sale 1999) due to direct mechanical damage and breakage of vegetative parts or indirectly via soil damage (Sun & Liddle 1993a; Cole 1995; Yorks *et al.* 1997). In general, wetlands tend to be more sensitive to trampling as roots and rhizomes are easily crushed in wet soils (Yorks *et al.* 1997).

Like trampling, wrack was also a regular feature of the intertidal at Bellocchio during the summer, forming dense wrack mats over the saltmarsh vegetation. Macroalgal production of the lagoon was markedly variable between years, which has previously been observed elsewhere (Pregnall & Rudy 1985; Valiela 1997). Almost no wrack occurred in the San Felice "ambient" plots during the experiment period as this site experiences a larger tidal range and is situated beside a well-flushed tidal creek and channel. However, we observed wrack in other parts of Venice Lagoon where currents were lower. The deposition of macroalgae on top of saltmarsh vegetation creates shading disturbance and physical crushing of underlying vegetation which reduces vegetation growth (Hulzen *et al.* 2006). Wrack that eventually gets mixed into the soil via bioturbation and burial could promote vegetation growth by enhancing soil nitrogen (Rossi & Underwood 2002).

The current study was carried out over two years in order to capture the multi-year effects of treatments in these long-lived perennials (Rietsma, Monteiro & Valiela 2010). However, the positive synergistic effect of preventing trampling and wrack deposition did not carry into the second year of the experiment, likely due to large natural variations in *Spartina* shoot density which could have masked potential responses in the second year. This variability

can be seen in the differences in start and end shoot density in the un-manipulated "PC" and "ambient" plots in 2013 although all plots had started out with similar densities in 2012 (Figure A2). This variability may partly be explained by the inherent instability of *Spartina* tussocks in the pioneering low intertidal zone in the short-term; tussocks of *S. anglica* (a hybrid species related to *S. maritima*) was found to undergo sudden shifts between dense vegetation and bare sediment over the scale of years, potentially due to by strong feedbacks between vegetation, sediment and erosion around the tussocks (van Wesenbeeck *et al.* 2008). In addition, Adriatic saltmarshes are facing the press disturbance of lateral erosion on the saltmarsh seaward edge (Fagherazzi 2013) due to high rates of subsidence and eustatic sea level rise (Bondesan 1989; Cencini 1998).

Some regional morphological differences were found that were not expected; flower number was significantly higher in Venice compared to Bellocchio even though the total shoot number in Venice was lower, indicating a higher rate of flowering. Besides shoot density differences, *Spartina* in Venice were also significantly taller than in Bellocchio.

A4.4.9 Limitations

Since we had removed the fences in the intervening winter to avoid storm damage to plots, we cannot exclude that external factors outside of the experimental design could have caused the unexpected reduced shoot densities in the ambient wrack deposition only plots. The study would have benefitted from higher levels of replication, although this was not feasible in this small Bellocchio Nature Reserve which has limited *Spartina* colonies.

A4.4.10 Conclusion

Through field testing, we found that the joint prevention of two stressors can result in significant improvements in vegetation growth where the prevention of one or the other stressor did not lead to significant improvements. In our experiment, the two co-occurring stressors of trampling and wrack disturbance are locally relevant stressors that are amenable to local management. Our results also underscore the importance of field experiments in identifying potential synergies between management actions.

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A4.6 Appendix

Pre-test determination of wrack cover on *Spartina* zone

To determine the conversion curve of fresh to dry macroalgal biomass, we collected fresh macroalgae samples from the Bellocchio Lagoon in varying weights. We strained each fresh macroalgae sample with a colander and weighed it in the field. We then dried the samples in an oven at 80°C for five days until constant weight. The wet weight was plotted against dry weight to obtain the following wet-to-dry weight conversion (Figure A1):

> Conversion formula:
>
> Macroalgal wrack dry weight= 0.1053 * macroalgal wrack wet weight -1.2926

To estimate the average macroalgal wrack dry biomass being deposited in the *Spartina* zone, we collected wrack from three 10cm x 10cm replicates sampled within twenty random 1m^2 square plots along the *Spartina* zone in Bellocchio in June and July 2012. We washed and sorted the wrack to remove non-macroalgal material (e.g. sand, shells and debris). We then dried the wrack at 80°C for five days until constant weight. We determined the dry macroalgal wrack deposit to be equivalent to 305 g/m^2. Based on the conversion formula, this corresponds to a dose of fresh macroalgae of 2.9 kg/m^2 .

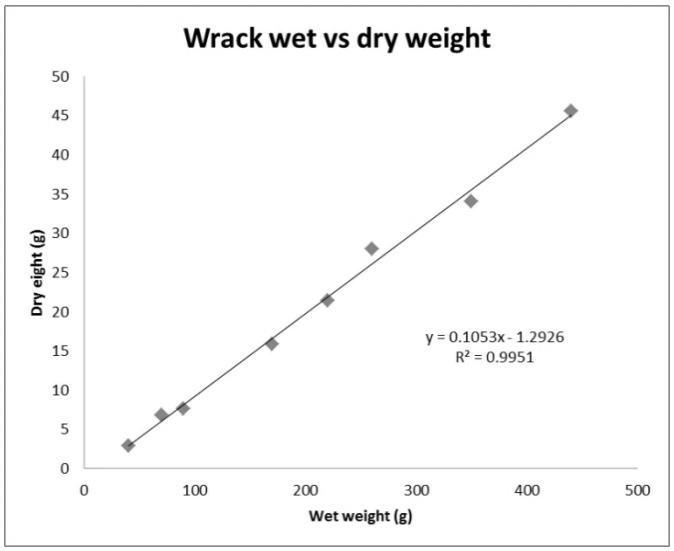

Figure A1 Wet-to-dry weight conversion of fresh macroalgae. Fresh macroalgae of various weights between 40 – 440g were collected from Bellocchio Lagoon. The fresh macroalgae was drip dried for 10 seconds and weighed in the field. Following this, the fresh macroalgae was brought to the lab where it was washed under running water for 10 second and then oven-dried at 80°C for five days and then weighed.

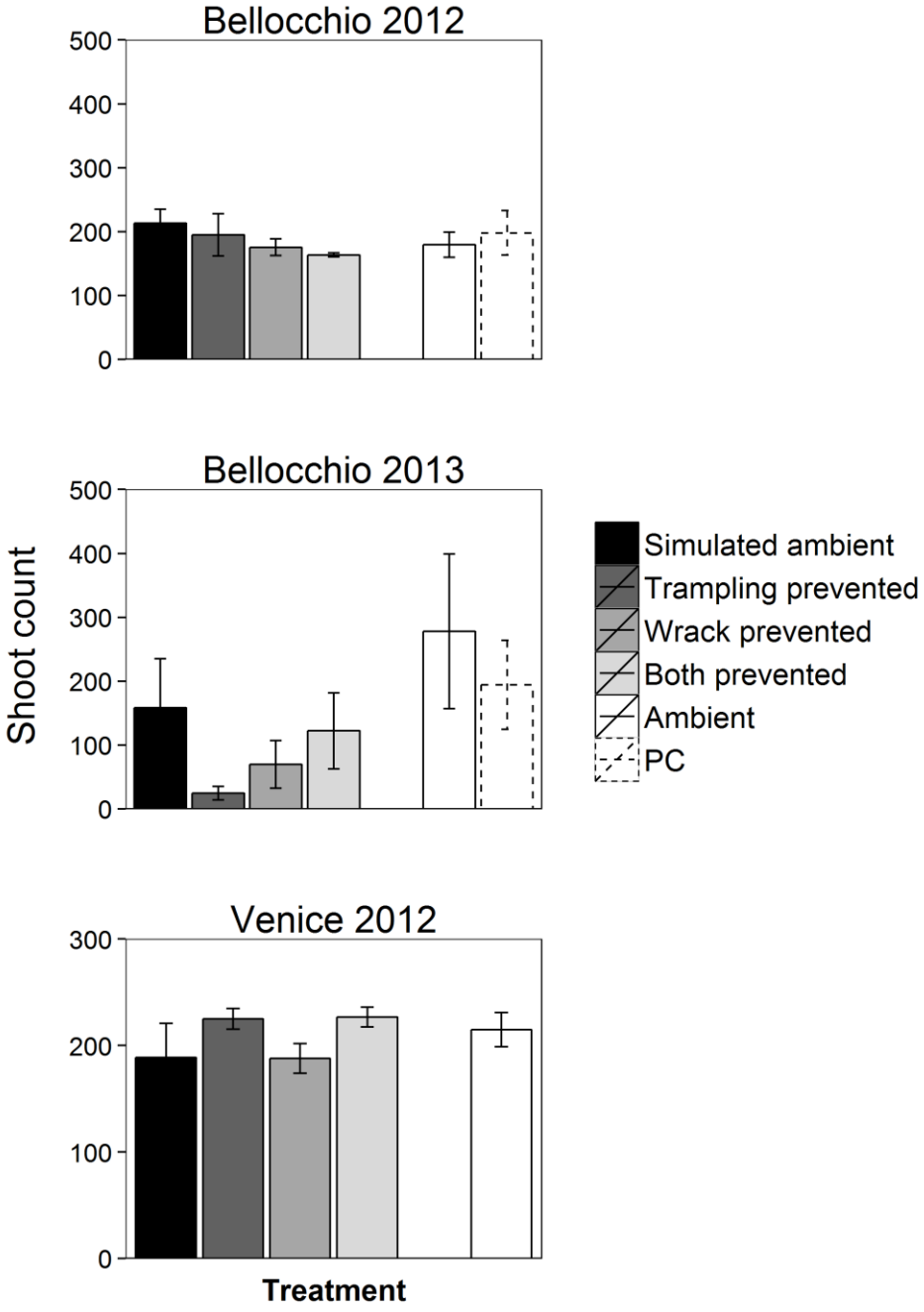

Figure A2 Mean shoot number (+/-SE) in Bellocchio (2012 and 2013) and Venice (2012) at the start of the growing season (June/ July). Treatments are: simulated ambient, trampling prevented, wrack prevented, both stressors prevented, ambient and procedural control. Note the differing y-scales between Bellocchio and Venice.

Article 5 Potential rapid displacement of native *S. maritima* by cryptic, non-indigenous *Spartinas* in north Adriatic saltmarshes

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Abstract

Understanding the patterns and dynamics of biological invasions at regional scales is crucial to predicting and mitigating their potential ecologic and economic impacts. *Spartina anglica* is a prolific colonizer that has invaded many saltmarshes worldwide, aided by deliberate introductions, with serious ecological effects. In Europe, its current known range extends from northern France to northern Sweden. A small patch of this species was recorded for the first time in Venice lagoon in 2003, following a large-scale dieback of the native *Spartina maritima*, but since then the distribution of either species has not been monitored. We identified, mapped and quantified the presence of native *S. maritima* and non-indigenous *Spartina* at six sites along about 400 km of the north Adriatic coastal lagoons. Identification was done using a two-step process using DNA analysis (chloroplast and ITS sequence polymorphism) and ploidy analyses. *S. anglica* and *S. townsendii* were unexpectedly widespread in the area, being well established virtually along the whole study region. The non-indigenous species was nearly indistinguishable from the native species in growth form, or patterns of spatial distribution.

A5.1 Introduction

Spartina is a small genus of halophytes that are widely found in mid- to low-intertidal areas of saltmarshes worldwide (Strong & Ayres 2013). Various species of the *Spartina* genus are notorious invaders, causing ecological damage by modifying the physical condition and trophic interactions of the native saltmarsh (e.g. Hedge & Kriwoken 2000; Castillo *et al.* 2000; Hacker *et al.* 2001; An *et al.* 2007; Grosholz *et al.* 2009; Pickart 2012). *Spartina* species can become invasive when introduced to intertidal areas outside of its original range (e.g. *S. densiflora* in Spain), or may hybridise with other closely-related *Spartina* species to create gregarious invasive hybrids (e.g. *S. anglica* arising from introduced *S. alterniflora* and native *S. maritima* in the UK). *Spartina* can spread via vegetative clones or seeds if the species is fertile (Strong & Ayres 2013).

S. alterniflora was introduced to Hythe, Southampton from north America around 1870 where it hybridized with the native *S. maritima* to form the infertile hybrid *S. townsendii* (Marchant & Goodman 1969). Chromosome doubling of *S. townsendii* subsequently led to the formation of the *S. anglica*, which is a fertile hybrid. Ten years later, *S. anglica* was observed to have replaced *S. maritima* which occurs in the mid- to low-marsh in the southern and eastern England coast as well as colonised areas of saltmarsh not occupied by its parents (Ferris, King & Gray 1997). However, as far as we are aware of, no study has looked at the competitive interaction between *S. maritima* and its hybrids; the replacement of *S. maritima* by *S. anglica* is thought to have occurred where the original cover was initially lost as a result of erosion from sea level rise, resulting in an elevation unsuitable for *S. maritima* (Ferris *et al.* 1997). The subsequent rise of *S. anglica* to notoriety as one of the world top 10 "Worst Invaders" by the World Conservation Union was facilitated by its natural fecundity, and by active introductions worldwide to facilitate coastal accretion (see Nehring & Hesse (2008) for a concise history of *S. anglica* invasion in Europe). In Europe, *S. anglica* has taken over large areas of saltmarsh in England and the Wadden Sea, with ranges extending north up to Sweden and west to northwestern Spain (Nehring & Adsersen 2006). Its southern range is presumably limited by high winter temperatures, while northern expansion is limited by frost damage in winter (Ranwell 1967).

S. maritima is the only native *Spartina* in the European and Mediterranean low-intertidal. *S. maritima* colonises new areas mainly by vegetative reproduction. It plays an important role as a lower marsh colonizer, initiating soil accretion that subsequently modifies soil properties and elevation in such a way as to facilitate the growth of other saltmarsh species. It is considered at risk as it is being displaced by *S. anglica* and *S. townsendii* in its northern limit and by *S. densiflora* in the southern Spanish limit (Sanchez & SanLeon 2001). The perennial *S. maritima* plays an important role in stabilizing the soil in the lower marsh (Cazzin *et al.* 2009). A worrying trend of

S. maritima regression has been documented in Venice Lagoon between 2002 and 2007, particularly at saltmarsh platform edges under strong tidal regimes (Cazzin *et al.* 2009; Mion D. *et al.* 2010). In 2002 and 2003, a previously un-documented non-indigenous *Spartina* was discovered and subsequently mapped in the lagoon; these small, diffuse patches were identified to be the infertile *S. townsendii* (Scarton *et al.* 2003, 2004). However, it was later reported to be potentially *S. anglica* (Cazzin *et al.* 2009). The appearance of non-native *Spartina* in Venice Lagoon has led to concerns that it could incur into *Zostera noltii* sea grass beds (Scarton *et al.* 2004). The concurrent large-scale regression of *S. maritima* in Venice Lagoon and the discovery of the non- native *Spartina* throughout Venice Lagoon could be related, perhaps due to a replacement process similar to the replacement of *S. maritima* by *S. anglica* in the UK.

There is currently no basic information regarding the presence and overall distribution of non-native versus native *Spartina* in Adriatic saltmarsh habitats. We undertook this large-scale survey of the major lagoons and saltmarsh areas to address this gap in knowledge. The target of the sampling was to get a high-level picture of distribution of non-native *Spartina* in the region.

A5.2 Methods

A5.2.1 Study species and area

Sampling was conducted in the lagoons of Bellocchio, Vallona, Caleri, Chioggia, Venice and Grado in Italy and from Seca in Slovenia (Figure 1). These saltmarshes were chosen to maximise the geographic coverage of the survey, to include the most northern and southern populations of *Spartina* on the Adriatic. Saltmarshes in this area have similar species distributions, mainly made up of *S. maritima* and *Salicornia veneta* in the lower intertidal, followed by mixed assemblages of *Limonium narbonense, Sarcocornia fruticosa, Juncus maritimus, Puccinellia palustris, Inula crithmoides, Halimione portulacoides, Suaeda maritima, Arthrocnemum macrostachyum* and *Aster tripolium*. *S. maritima* was also observed to occur beside creeks and in internal depressions on the saltmarsh flat in mixed assemblages of *L. narbonense* and *A. tripolium* in Venice, Grado and Chioggia. The three *Spartina* species share many similar morphologies and habitat preference (Goodman *et al.* 1969; Marchant & Goodman 1969). Separate studies on *S. maritima* (Castillo *et al.* 2005) and *S. anglica* (Hubbard 1991) have found these species to have high levels of phenotypic plasticity, with plant morphologies determined by factors like soil condition and colony age. Although the *S. anglica* is often noted to grow larger, a definitive test would require the examination of chromosome numbers of specimens (Goodman *et al.* 1969).

Figure 1 Location of survey sites in the Adriatic.

A5.2.2 Field sampling

In June, July and September 2014, individual *Spartina* colonies were sampled in a haphazard manner in the study lagoons, with the general aim of collecting samples from as many colonies over the largest area possible per survey day. One *Spartina* plug (i.e. shoots with intact root mass approximately the size of a tennis ball) per colony was collected from the low-intertidal and interior saltmarsh flats. Special attention was paid to collect samples from *Spartina* colonies adjacent to each other that exhibited apparently different morphologies (e.g. in leaf size and colour). Each colony was marked using a handheld Garmin GPS 76Cx unit to allow researchers to locate the colonies again.

Each lagoon was surveyed for one day for approximately 2.5hours on either side of the low tide. Researches accessed Grado and Venice lagoon saltmarshes by boat while the other lagoons were accessed from land by foot. Distances covered on days sampling was done by boat were larger than those by foot. All collected plugs were transported to the laboratory in Ravenna, Emilia-Romagna, where live green shoots were collected and freeze dried for chloroplast and ITS DNA analysis. Live *Spartina* samples were kept alive until transport to Ghent University, Belgium, for the flow cytometer analysis.

A5.2.3 Species identification general approach

In order to differentiate between species, we used a combination of two different approaches: sequences polymorphism of the *trnT-trnL* of chloroplast DNA (cpDNA) and a fragment of the Internal transcribed spacer (ITS), and ploidy analyses. We sequenced the cpDNA trnT-trnL spacer as it is the most variable non-coding sequence thus far used with 3 known fixed variable sites to distinguish between *S. maritima* and *S. alterniflora*-type chloroplast (Baumel, Ainouche & Levasseur 2001; Baumel *et al.* 2002), while the ITS spacer is frequently used in phylogenetic studies of related species due to its fast evolutionary rate and ease of amplification (Baumel *et al.* 2002). The combination of molecular genetics and ploidy analysis is required to differentiate between allopolyploid hybrids and their parent plant species due to similarities in chloroplast and nuclear DNA arising from hybridization and chromosome doubling. *S. townsendii* and its allopolyploid, *S. anglica*, possess the same chloroplast and nuclear DNA and differ only in the amount of DNA material per cell; the chloroplast DNA (cpDNA) is maternally inherited from *S. alterniflora*. Thus, ploidy analysis is required together with chloroplast DNA analysis to distinguish between the two hybrids and the paternal *S. maritima* (Figure 2) (after Baumel *et al.* 2001). With this suite of analyses we are not able to distinguish between *S. townsendii* or *S. alterniflora*, although there is cause to suspect that *S. alterniflora* is present in the Adriatic.

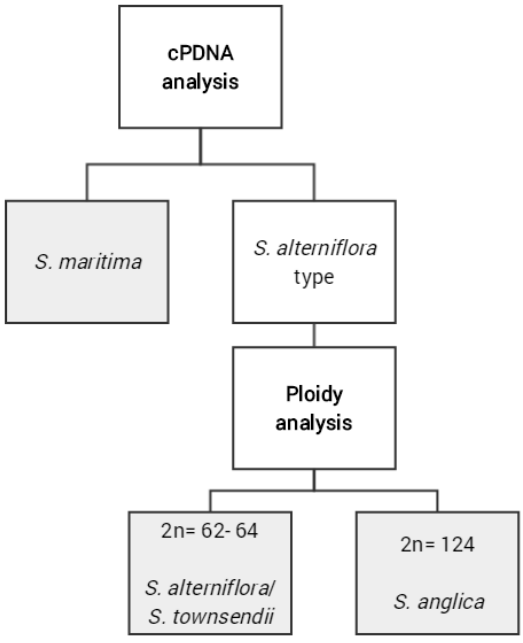


Figure 2 Chloroplast DNA and ploidy analysis to distinguish between related species of *Spartina*

Chloroplast DNA sample preparation and analysis

Genomic DNA was isolated from leaves using Phire Plant Direct PCR Master Mix (Thermo Scientific) and products were amplified using polymerase chain reactions (PCR). Before using specific *Spartina* primers, *Spartina* leaves were checked for suitability for direct PCR using control primers supplied with the Master Mix following the manufacturer's instructions. Each sample (a punch of leaf of approximately 2mm in diameter) was placed in 20µL of dilution buffer and crushed with a pipette tip by pressing briefly against the tube wall. An amount of 0.5µL of the supernatant was used as template for a 20µL PCR reaction. Each 20µL reaction contained 0.5µM of primers and 1.5mM $MgCl_2$. The *trnT-trnL* spacer was amplified with primers B48577 and AG9291 of Taberlet *et al.* (1991). The PCR cycling regime involved 30 cycles of 5min denaturation at 98°C, 5sec annealing at 54 °C, and 1min extension at 72°C. PCR products were checked on 1.5% agarose gels before sequencing. Purification and sequencing of PCR products in both directions for each region was conducted by a certified laboratory, Macrogen, in South Korea. Sequences obtained were edited and aligned the manually using BioEdit version 7.0.4 (Hall 1999). Sequences were first aligned with ClustalX (Thompson *et al.* 1997) embedded in BioEdit. Consensus sequences were checked against available sequences on Genbank: *S. maritima* (AF275669), *S. anglica* (AF275668) and *S. alterniflora* (AF275667) for the *trnT-trnL* spacer (Baumel *et al.* 2001).

The number and position of variable sites and number of haplotypes were calculated for each molecular marker by using DnaSP v. 4.50.3 (Rozas *et al.* 2003). Diversity values and relationships between the haplotypes were calculated using median joining with the software Network v. 4.6.1.1 (Bandelt, Forster & Rohl 1999), unrooted haplotype networks were used to visualize the results.

ITS sample preparation and analysis

The procedures for DNA extraction, amplification and sequencing for the ITS regions is similar to that for the cpDNA with slight modifications. The primers *ITS-1* and *ITS-4* were used to amplify the ITS region (White, Bruns & Taylor 1990). The products were annealed at 48°C and consensus sequences were checked against available sequences on Genbank: *S. maritima* (AF272776) and *S. alterniflora* (AF272775) (Baumel *et al.* 2001).

Ploidy level analysis

Flow cytometry provides an efficient method to estimate the amount of nuclear content in plant DNA and thus the identification of ploidy level (Arumuganathan & Earle 1991). A suspension of whole plant nuclei is first obtained from fresh plant material via careful chopping

in a suitable buffer. The suspension containing the stained nuclei is analysed in a flow cytometer where cells are passed single-file before a light beam and the intensity of fluorescence from the stained nuclei is measured. An estimate of nuclear DNA content is made by comparing the fluorescence intensity of the sample with fluorescence from a DNA standard or reference plant of known genetic mass (Arumuganathan & Earle 1991).

Nnuclei from fresh *Spartina* samples was extracted using the Galbraith *et al.* (1983) method. Fresh, green leaves from *Spartina* samples were chopped with a sharp razor blade in 300µl Galbraith's buffer and filtered through a 40µm nylon mesh. The filtrate was stained with propidium iodide (final concentration 10µM) and analyzed using the flow cytometer Epics Altra, Beckman. Diploid *Arabidopsis thaliana* was used as a calibration standard, and calibration samples were run between every 3 to 5 *Spartina* samples. *S. maritima* (2n = 60) and *S. townsendii* (2n = 62) are known to fluoresce approximately the same amount, while *S. anglica* (2n= 124) fluoresces at twice the amount (Baumel *et al.* 2003).

Plant morphology

Observations were made regarding the general growth structure of tussocks from where specimens were collected, including growth form, location and potential defining characteristics. Leaf width of the largest leaf per plug was measured to determine its usefulness as a rapid field-based indicator for species identification. We modeled leaf width with factor species (3 levels: *S. maritima*, *S. anglica*, *S. townsendii*) using a linear model ("lm" function in the "lme4" package) in R version 3.0.3 (R Core Team 2014).

A5.3 Results

A total of 111 plants were collected during the survey (Table 1). However, only 59 were positively identified due to a combination of unforeseen circumstances. Due to waiting time associated with transport and laboratory waiting time, some plant conditions deteriorated and were in too poor condition to be tested. This was particularly detrimental for the ploidy analysis, which required fresh, green leaves in good condition (Baumel *et al.* 2002). In addition, some cpDNA sequences could not be aligned and were inconclusive. However, we do not think that this biased our results in terms of potential differential susceptibility of the different species since all three species were well-represented in the final results.

Table 1 Tally of samples collected per lagoon and number positively identified for species.

Site	Samples collected	Sampled positively identified
Bellocchio	23	13
Vallona	2	1
Caleri	18	8
Chioggia	15	8
Venice	22	10
Grado	29	17
Slovenia	2	2
Grand Total	111	59

In some cases, only one of the analyses (cpDNA or ploidy level) returned a positive result out due to inconclusive DNA sequences in the case of cpDNA, or bad leaf quality in the case of ploidy. For these samples, species could be identified if:

- The cpDNA indentifies the sample as *S. maritima*
- The ploidy analysis results show 2n=124 (which can only happen in the case of *S. anglica*)

A5.3.4 Species abundance and distribution

Percent *S. maritima* occurrence among fully identified samples was highest in Slovenia and Chioggia (100%), followed by Venice (73%), and Grado (18%). None of the samples from Bellocchio and Caleri were identified as *S. maritima*. One individual of *S. anglica* was identified in Vallona. Non-native *Spartina* was not limited to individual lagoons, and did not depend on lagoon size and latitude (Figure 3 to 5). However, we note that this is a preliminary survey and the survey attempted to provide a broad overview of species at the survey sites and not designed to be a quantitative representation of the distribution of the three species.

(A)

(B)

(C)

Figure 3 *Spartina* species identified at the (A) Bellocchio Lagoon, (B) Vallona and (C) Caleri Lagoon study site. Base map shown is from the ArcGIS Server World Imagery (sources: Esri, DigitalGlobe, Earthstar Geographics, CNES/Airbus DS, GeoEye, USDA FSA, USGS, Getmapping, Aerogrid, IGN, IGP, swisstopo, and the GIS User Community).
Spartina species identified at the Caleri Lagoon study site.

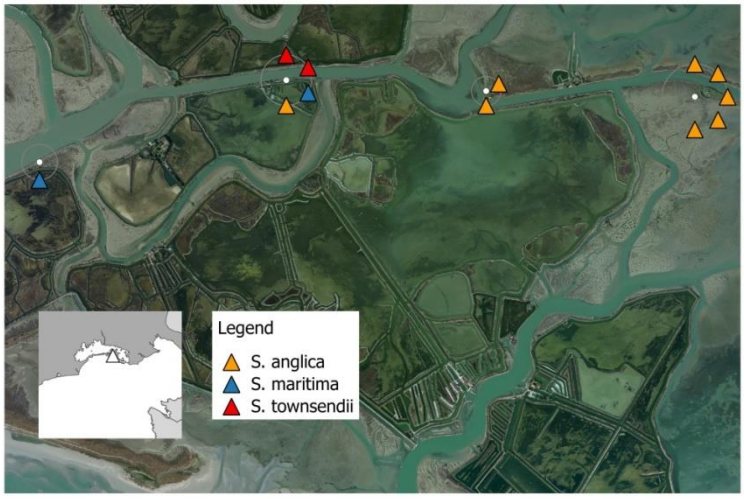
(A)

(B)

(C)

Figure 4 *Spartina* species identified at the (A) Chioggia, (B) Venice (San Felice) and (C) Venice (Treporti) study site. To avoid overlap, data points in some maps were set to displace around the central point (white circle). Base map shown is from the ArcGIS Server World Imagery (sources: Esri, DigitalGlobe, Earthstar Geographics, CNES/Airbus DS, GeoEye, USDA FSA, USGS, Getmapping, Aerogrid, IGN, IGP, swisstopo, and the GIS User Community).

(A)



(B)

(C)

Figure 5 *Spartina* species identified at the (A) central and (B) east Grado, and (C)Seca San Giorgio canal, Slovenia study site. To avoid overlap, data points in some maps were set to displace around the central point (white circle). Base map shown is from the ArcGIS Server World Imagery (sources: Esri, DigitalGlobe, Earthstar Geographics, CNES/Airbus DS, GeoEye, USDA FSA, USGS, Getmapping, Aerogrid, IGN, IGP, swisstopo, and the GIS User Community).

A5.3.5 Ploidy level

The 2n fluorescence peak for each *Spartina* sample was compared to the 16n fluorescence peak of *A. thaliana* reference samples; this ratio was approximately 1.5 for *S. maritima* or *S. townsendii* and approximately 3 for *S. anglica*. As expected, *S. anglica* nuclei had approximately twice the amount of DNA compared to the *S. maritima* and *S. townsendii* nuclei (Figure 6). A total of 30 samples were identified as 2n= 124 and 20 were identified as 2n= 60- 62. Ploidy level could not be determined for some samples as these samples repeatedly gave broad or indistinct peaks, most likely due to sub-optimal condition of leaves.

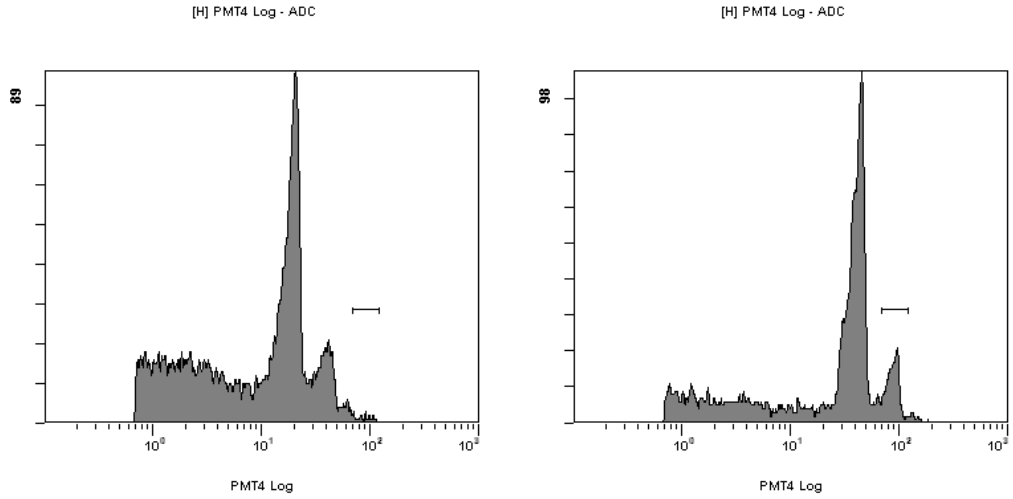


Figure 6 Example output graph showing nuclei DNA content. The Y-axis represents the number of nuclei and X-axis represents the log value of relative DNA content (at the PMT4 channel). (A) shows a typical *S. maritima*/ *S. townsendii* cytometer output where the 2n peak occurs at ≈20; (B) shows a typical *S. anglica* cytometer output where the 2n peak occurs at ≈40.

A5.3.6 Genetic variability of the trnT – trnL region of the cpDNA

Fifty-one sequences of the trnT – trnL region were compared with the sequences from AF275669 and AF275667 over 690 bp. Results showed the presence of 590 aligned basepairs with seven nucleotide substitutions and ten INDELs (Table 2). Three sites were parsimony-informative (298, 492, 541) and corresponded to the polymorphic sites between *S. alterniflora* and *S. maritima* as observed by Baumel *et al.* (2003). Seven different haplotypes were identified (Table 3). Hap 3, Hap4 and Hap5 correspond to *S. maritima*, while Hap 1, Hap2, Hap6 and Hap7 correspond to *S. alterniflora*-type chloroplast. The haplotype network showed the presence of two main haplotype (Hap2 and Hap3) surrounded by two and three private haplotypes respectively with a clear distinction between the two maternal sequences (Figure 7).

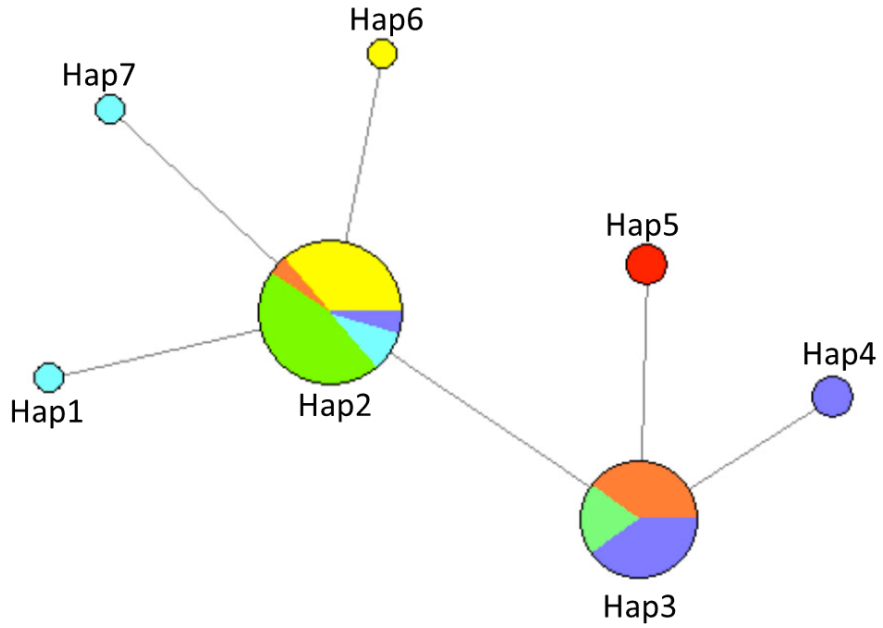


Figure 7 Network diagram of haplotypes of the trnT – trnL region of *Spartina* spp. Colours represent lagoons where the haplotype was collected.

Table 2 Polymorphic sites of the seven haplotypes of the trnT – trnL region of *Spartina* spp. checked against consensus sequences available on Genbank: *S. maritima* (AF275669), *S. anglica* (AF275668) and *S. alterniflora* (AF275667). Dots (.) indicate identical bases, dashes (-) indicate INDELs. The three parsimony-informative sites of Baumel *et al.* (2003) are shown in grey.

	Nucleotide position																	
	176	**298**	**492**	**516**	**517**	**542**	**558**	**570**	**572**	**590**	**613**	**625**	**645**	**649**	**650**	**657**	**663**	**681**
Hap1	G	G	T	A	-	G	A	A	G	A	T	A	A	A	A	A	A	A
Hap2	A	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap3	A	T	G	.	-	T	.	.	.	.	.	.	.	.	.	.	.	.
Hap4	A	T	G	-	-	T	.	.	.	.	.	.	.	.	.	.	.	.
Hap5	A	T	G	.	A	T	.	.	.	.	.	.	.	.	.	.	.	.
Hap6	A	.	.	.	-	.	.	.	.	.	.	.	.	.	-	.	.	.
Hap7	A	.	.	.	-	.	T	.	A	-	-	-	-	-	.	-	-	-
AF275667	A	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.
AF275668	A	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.
AF275669	A	T	G	.	-	T	.	G	.	.	.	.	.	.	.	.	.	.

Table 3 Species frequencies of each *Spartina* at the 6 saltmarshes identified by joint analysis of cpDNA and ploidy level. * Some individuals identified as Hap_2 had inconclusive ploidy results, these are indicated in brackets () next to the confirmed species frequency based on the joint analysis. **For 12 samples, identification of *S. anglica* was done with ploidy analysis only as the DNA sequence for these samples were inconclusive.

Species & haplotype	Site						
	Bellocchio	Vallona	Caleri	Chioggia	Venice	Grado	Slovenia
S. anglica							
Hap1	0	0	1	0	0	0	0
Hap 2	5 (1)*	0	1 (2)*	0 (1)*	0	8	0
Hap 6	1	0	0	0	0	0	0
Hap 7	0	0	1	0	0	0	0
From ploidy analysis only**	3	1	4	0	2	3	0
S. maritima							
Hap 3	0	0	0	7	6	3	0
Hap 4	0	0	0	1	1	0	0
Hap 5	0	0	0	0	0	0	2
S. townsendii							
Hap 2	4	0	1	0	1	3	0

A5.3.7 Genetic variability of the ITS1 region

The total length of the aligned sequences was 627bp. Since the ITS region is one of the most frequently used sequences in plant phylogenetic studies due to its relatively fast evolutionary rate. Baumel *et al.* (2001) found 48 nucleotide differences between *S. maritima* and *S. alterniflora* for 600 bp sequenced, and reported that the hybrid *S. anglica* possessed both these parental ITS sequences (as detected via restriction fragment length polymorphisms). Therefore, we expected to obtain both parental ITS sequences in *S. anglica*. However, no heterogeneous amplified ITS fragments were observed; all sequences corresponded to *S. maritima* (AF272776; Baumel *et al.* 2001) (data not shown). One possible explanation could be that homogenization of rDNA (concerted evolution) after hybridization led to the loss of one parental rDNA (i.e. that of *S. alterniflora*), as was the case with allopolyploid hybrids of *Tragopogon* (Kovarik *et al.* 2005).

Another possible reason we did not find heterozygotes individuals could be that there was a biased sampling of one parental type or that we obtained a bad hybrid sequence that could not be read due to nucleotide polymorphisms. Future work with ITS sequencing could benefit from cloning of amplified sequences before sequencing. Moreover, a new sampling with a larger number of individuals could increase the chance of capturing both parental types.

A5.3.8 Plant morphology

Our observations were in line with others who reported that the three species display high morphological variability in terms of plant and leaf morphologies with overlapping morphological ranges (Goodman *et al.* 1969; Marchant & Goodman 1969). We found that leaf width of *S. anglica* (mean ± S.E.: 5.65 ± 0.21, range: 3.5 - 8.5 cm) and *S. townsendii* (mean ± S.E.: 5.25 ± 0.31, range: 4.0 - 6.5 cm) were not significantly different to *S. maritima* (mean ± S.E.: 5.56 ± 0.21, range: 4.0 - 7.5 cm) and is thus not likely to be a good predictor of species in the field (LM: $F(2, 47)=0.5005$, $P=0.6094$).

A5.4 Discussion and conclusion

This work represents some of the first genetic studies on *S. maritima* (and related hybrids) in the region. The spread of non-native *Spartina* to the main lagoons of the Adriatic was an unexpected outcome. We did not sample any *S. maritima* individuals at the Caleri and Bellocchio sites and only one individual of *S. anglica* was found in Vallona. The spread of non-native *Spartina* seems to have occurred within a decade since the first sighting in the Venice Lagoon in 2002 (Scarton *et al.* 2003, 2004). The ability of *S. anglica* (and potentially also *S. townsendii*) to out-compete native saltmarsh species (Reeder & Hacker 2004; Nehring & Hesse 2008), convert open mudflat habitats to dense monocultures (Reeder & Hacker 2004) and even incur into seagrass and *Salicornia* habitats (Loebl, Van Beusekom & Reise 2006) is a potential cause for concern in the study lagoons. We currently do not know if invasive *Spartina* are out-competing the native *S. maritima* or other low-intertidal species like *Salicornia veneta* in our study area. It is equally unclear what higher-order effects (if any) this would cause in this ecosystem. The overall picture of risks and benefits of *S. anglica* invasion is currently unclear as data on the species is patchy (Nehring & Hesse 2008). The invasion pressure from non-native *Spartina* could interact with other global stressors like sea level rise and increasing average temperatures to cause large-scale alterations to the native saltmarsh assemblage.

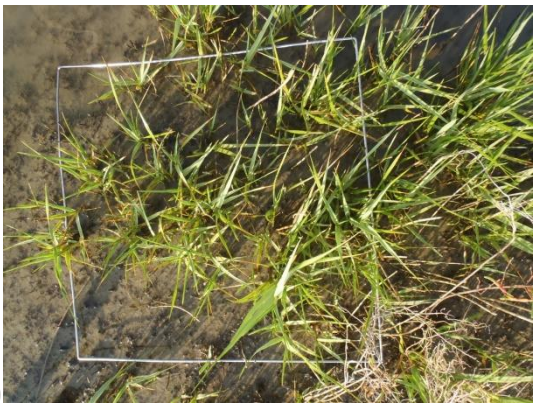
Further understanding of the ecology and invasion history of *S. anglica* in the Adriatic is necessary. If it is a relatively new alien species that could potentially have an invasion trajectory

like the populations in northern Europe and the USA, then early detection and eradication would be needed to stop its spread as it is highly invasive and resistant to mechanical removal efforts (Dethier & Hacker 2004). However, it could also be an 'old' alien species that has existed side-by-side with native *Spartina* and has a lower level of invasiveness due to the specific physical conditions in the Mediterranean (e.g. microtidal, warm winters etc.); in this case control and eradication would be less pressing.

Our observations indicate that the three species of *Spartina* are morphologically highly variable within species and are largely indistinguishable. For example, we found all species to grow in both monospecific tussocks (of various dimensions) as well as within mixed saltmarsh assemblages of *Limonium*, *Aster* and *Sarcocornia* and there were no differences in leaf widths. Scarton *et al.* (2003) reported that the non-native *Spartina* in Venice Lagoon was bright green, growing in dense tussocks with tall shoots, which visibly distinguish it from the native *S. maritima*. However, in our survey, *S. townsendii* and *S. anglica* growing on saltmarsh borders generally formed small tussocks and did not exhibit excessively large culms. Furthermore, the size of tussocks is likely to vary largely depending on the age of the colony. Although not measured, the general aspect of the plant (i.e. narrow or wide form) could be a useful indicator of species, as *S. maritima* culms tended to be narrow with leaves close together while *S. townsendii* and *S.anglica* tended to have a wider growth form, although we note that there were also exceptions to this rule (Figure 8).

Our results reflect other studies (e.g. Yannic, Baumel, & Ainouche (2004)) that report on low genetic variability in *S. maritima*. The presence of what we consider to be *S. townsendii* was unexpected and more work should be done to determine if this is the same species that arose in the UK and was subsequently spread to the Adriatic or if it occurred from independent hybridization events. The latter was the case for *S. neyrautii*, an *S. maritima* x *S. alterniflora* hybrid like *S. townsendii*, which was shown to have arisen independently in France (Baumel *et al.* 2003; Salmon, Ainouche & Wendel 2005). We note that we did not consider the possibility that the original *S. alterniflora* would be present in our study area, and our testing methods do not allow us to distinguish between *S. alterniflora* and *S. townsendii*. This assumption warrants further study.

(A)  (B) 

(C)  (D) 

(E) 

Figure 8 Photographs showing (A) *S. maritima* with the "narrow" growth form (indicated by arrow) in mixed assemblages of *Sarcocornia* and *Aster* on the saltmarsh platform, (B) *S. anglica* patch showing the "wide" growth form, (C) *S. anglica* patch without the "wide" growth form, (D) *S. townsendii* colony showing the "wide" growth form and (E)*S. maritima* apparently showing both "narrow" and "wide" growth forms.

A5.4.9 Suggested future work

Future work could further explore the potential for using a multivariate approach to discerning species by using multiple physiological traits and associated abiotic conditions like soil type and redox. Further, DNA analysis with microsatellites developed for *S. alterniflora* in China (Guo *et al.* 2015) could provide clues of invasive *Spartina* phylogeography in the region, as well as potentially provide a simpler procedure to determine between species, which would be an improvement from the current two-step procedure. Analysis of historical herbarium samples could also yield temporal information as to whether this is a relatively new invasion that could worsen, or if *S. anglica* is an old invasive that has not managed to establish large meadows under local environmental conditions.

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Chapter 4 Final Perspectives, limitations and future work

In this chapter, I outline the work done in each article, summarise the main conclusions and highlight unexpected findings and challenges faced (Table 4.1). I further discuss the contribution of the findings, point out limitations and suggest directions for future research.

4.1 Final perspectives

In Article 1, I conducted a preliminary review of tools and techniques for cumulative stressor detection, mapping and prediction of interactions which set the scene for developing the work plan for this thesis. Tool/ technique selection depends on analysis goals, data availability and time investment. To build upon existing empirical knowledge of stressor interactions, I decided to conduct a systematic review of studies that manipulated two or more stressors orthogonally on saltmarsh vegetation in order to draw general conclusions about interactions between specific pairs of stressors. The aim was to combine this with a spatial analysis of stressors on Adriatic saltmarsh habitat to predict areas that are particularly vulnerable to stressors and their interactions. I also decided to investigate stressor interactions via empirical experiments as these provide basic understanding of processes that is still largely lacking. Empirical data provide important input for the building of prediction tools.

In Article 2, I used a combination of techniques to relate results from a systematic review with a cumulative stressor spatial analysis in order to identify (1) existing hotspots of stressors, (2) potential synergistic interactions between stressors and (3) gaps in knowledge with regards to stressors and their effects on Adriatic saltmarsh systems. The results showed that local saltmarshes face a combination of large-scale drivers like climate change, sea level rise and meteorological processes, as well as local-scale drivers related to river alteration, dredging, clam farming and vessel transport. I identified areas with each lagoon that are relatively more impacted than others; however, comparison between lagoons was hampered by patchy data availability. The systematic review indicated that two important stressors, salinity and increased inundation, could potentially have negative synergistic effects on Adriatic saltmarshes as the lagoons convert into more open marine systems due to relative sea level rise. This analysis also identified a lack of publicly-available drivers and pressures data, particularly in the individual lagoons in the Po Delta.

By simulating different management scenarios using field experiments in impacted saltmarshes, I developed new empirical knowledge that contributes to our current understanding of management effects in multiple stressor scenarios. In particular, for Article 3, I found that saltmarsh vegetation would be more drastically affected by increased inundation in low than in

Table 4.1 Outline the work done in each article

Articles	Key research question	Method	Results and conclusions	Unexpected findings/ challenges
Article 1 *"Qualitative and quantitative methods for evaluation of interactions between stressors"*	• What are methods employed to investigate stressor interactions?	Literature review	• The main methods for detecting and quantifying interactions are experimentation and regression tree analysis; prediction methods include modelling and Bayesian network analysis.	-
Article2 *"Assessment of vulnerability to multiple stressors: an example from north Adriatic saltmarshes"*	• What are multiple stressors that affect saltmarsh vegetation? • Do these stressors interact? • Are there hotspots of stressors in Adriatic saltmarsh habitats?	Systematic review and mapping	• Stressors in all the main saltmarsh lagoons are generally similar. • Three common adverse effects of stressors were: increased lateral erosion, increased waterlogging and loss of sediment from the system. • Many drivers of stressors are global (e.g. meteorological forcings, sea level rise etc.). • Increasing salinity and inundation time are potentially important synergistic stressors that could affect these saltmarsh habitats.	• Meta-analysis of specific stressor combination interactions was hampered as few orthogonal multiple stressor studies were found. • There are data gaps of stressor distributions.
Article 3 *"Nutrient levels modify saltmarsh responses to increased inundation in different soil types"*	• Do nutrient enrichment and increased inundation due to sea level rise interact with local soil conditions to affect saltmarsh vegetation?	Mesocosm experiment	• Increased inundation had a highly negative effect on *Spartina* growth • The effect of inundation was largest in high nutrient and low organic soil. • Response of saltmarsh vegetation to increased inundation will be site specific.	• *Spartina* sourced from different lagoons had markedly different survival, leading to the hypothesis that different species could be present in the study lagoons.
Article 4 *"Effects of prevention of co-occurring and interacting local stressors in depleted* Spartina *habitats"*	• Could co-management of multiple stressors in saltmarshes lead to better-than-expected outcomes?	Field experiment	• Prevention of human trampling and macroalgal wrack deposition together yielded beneficial effects on *Spartina* growth while prevention of only one stressor had no significant beneficial effects.	• Results from the second year of manipulation were less conclusive, most likely due to the strength of other uncontrolled factors that influence *Spartina* shoot densities in the time-scale of years.
Article 5 *"Potential rapid displacement of native* S. maritima *by cryptic, non-indigenous* Spartina *in north Adriatic saltmarshes"*	• Are Adriatic saltmarshes being invaded by hybrid Spartina?	Field survey and molecular genetic analysis	• Both *S. anglica* and *S. townsendii*, two invasive hybrids, were found to be present in the Adriatic. • There is high overlap in distribution of the species. • High phenotypic plasticity means that a positive ID currently requires molecular genetic testing.	• The presence of non-native *Spartina* was very widespread, being found in six out of seven sampling areas.

medium organic matter soils, and especially in estuaries already under high nutrient availability. This implies that management interventions aiming at enhancing the resilience of saltmarshes to future climatic changes will need to consider site-specific factors, such as soil type differences or the co-occurring effects of other stressors, such as high nutrient loads. In Article 4, I manipulated two existing stressors to simulate the joint effect of management, specifically the prevention of damage from recreational trampling and smothering by deposition of excess macroalgal wrack due to high nutrient loads. Results show that the removal of either stressor individually did not benefit saltmarsh vegetation growth, but the combined removal of both stressors was necessary to detect some benefits. Such synergistic interactions between management actions pose particular challenges, and highlight the need for field experiments simulating realistic management scenarios to guide effective remediation of existing impacts.

Experimental work related to Article 3 revealed unexpected responses from saltmarsh transplants in field experiments. These findings coupled with taxonomic observations led me to hypothesise that a previously unreported invasive *Spartina* could be present in the Adriatic saltmarsh systems. This hypothesis was confirmed following a large-scale survey and molecular testing which showed the presence of invasive *Spartina* (*S. townsendii* and *S. anglica*) in all the main Italian Adriatic lagoons (Article 5). An invasive *Spartina* sp. was recorded for the first time in 2002 in a few isolated patches in Venice but seems to have spread rapidly throughout the Adriatic lagoons, potentially exerting additional pressure on the already impacted systems.

Overall, this thesis provided relevant empirical information regarding interactions between specific stressors and between management actions, as well as discovered an invasive *Spartina* in the study site. The attempt to predict potential cumulative impacts from stressors and their interactions on saltmarsh vegetation was hampered both by the lack of spatial information regarding stressor distributions as well as empirical data from stressor interaction experiments. As such, the focus of this thesis was centred on detecting and developing empirical knowledge of interactions instead of prediction of interaction effects.

4.2 Limitations

Field experiments are powerful tools to test hypotheses about the effects of different management scenarios in ecosystems affected by multiple stressors. However the challenges of conducting field experiments can lead to focus on certain types of interventions, and clearly limit the spatial and temporal scales of the work. For example, stress by wrack in the North Adriatic lagoons is a consequence of excess nutrient load, which was impossible to experimentally reduce. As an alternative I focused directly on the removal of wrack accumulation, with the idea of

simulating one of the benefits that could derive from a management control of nutrient loads in these lagoons. Similarly, in the nutrient enrichment and inundation experiment, I placed increased inundation treatments 10cm below the natural *Spartina* elevation to simulate increased inundation resulting from sea level rise; admittedly, this cannot perfectly reproduce the projected effects of increasing sea levels which occurs gradually over a longer time period rather than in a step-wise manner. Still, this method can provide useful indications about what factors will enhance or reduce the sensitivity of the system to increased inundation events.

In the spatial analysis of cumulative stressors, the lack of publicly-available pressure and driver data was a large limitation in the multiple stressor spatial analysis. Available data was often available in image files that had to be digitized by hand; shapefiles would have provided much more information for analysis. Further, the amount of data, format of presentation and most likely collection between regions resulted in data that was difficult to compare across regions, for example, higher accuracy maps of saltmarsh vegetation were available from the Venice and Marano Grado lagoons but the saltmarsh habitats were classed in different vegetation cover categories such that this information had to be discarded for lower accuracy but more uniform Corinne land cover maps. The low overlap of stressor types identified in the systematic review and local stressors identified in the spatial analysis resulted in a reduced ability to draw inferences between the two components of the analysis.

4.3 Recommendations for future work

The discovery the non-native and potentially invasive *Spartina* spp. throughout the Italian Adriatic saltmarshes raises the need for increased research to determine the exact distribution of these non-native plants, and the ecological effects that could arise from their establishment, including potential interactions with other local stressors. However, since the native and introduced *Spartina* spp. have largely overlapping taxonomic features, more rapid testing methods need to be developed to facilitate rapid identification (e.g. for eradication purposes); the current two-step molecular testing procedure required to identify the species is overly time-and effort-intensive.

There is currently little available long-term spatial data for drivers and pressures affecting saltmarsh vegetation in the Adriatic lagoons. Existing habitat data between lagoons was significantly different, due to different naming practices and potentially survey methods. High resolution data of saltmarsh habitat is scarce, especially for the Po Delta Lagoons. This scarcity of data hinders the holistic assessment of multiple stressors of these highly threatened habitats. A better monitoring system to capture the spatial distribution of drivers, pressures and receptors is

needed to ameliorate the problem of lack of spatial data. Existing publicly-held environmental data should be made more easily accessible, preferably at a central database, to facilitate access and use for scientific analyses.

Although not conducted for this thesis, an analysis using Bayesian methods to estimate likelihood of outcomes for different areas of saltmarsh under different stressor combination scenarios could be feasible for this study area. Such an analysis would provide useful insights for prioritization of management actions or selection of restoration sites based on areas of saltmarsh most/ least resilient to stressors. Bayesian methods could be a promising tool due to its ability to handle patchy and uncertain data, as well as a mix of information types: i.e. data extracted from empirical experiments/ meta-analysis, results from physical models and expert opinion.

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Appendix- Published articles

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Nutrient levels modify saltmarsh responses to increased inundation in different soil types

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ABSTRACT

Saltmarshes have been depleted historically, and cumulative stressors threaten their future persistence. We examined experimentally how nutrient availability (high vs. low) affects the responses of *Spartina maritima* to increased inundation in two mineral soil types (low vs. medium organic). Increased inundation, one of the effects of accelerated sea level rise, had negative effects on most plant growth parameters, but the magnitude varied with soil and nutrient levels, and between plants from different locations. Average differences between inundation treatments were largest at high nutrient conditions in low organic matter soils. We conclude that saltmarsh vegetation would be more drastically affected by increased inundation in low than in medium organic matter soils, and especially in estuaries already under high nutrient availability. This knowledge enhances the prediction of changes at the foreshore of saltmarshes related to sea level rise, and the development of site-specific conservation strategies.

1. Introduction

Many valuable coastal ecosystems have declined due to the cumulative effects of local and global anthropogenic stressors (Halpern et al. 2008; Ban et al., 2014; Strain et al. 2014), leading to loss of valuable ecosystem services and contributing to persistent poverty and risk (Millennium Ecosystem Assessment, 2005). Over the centuries, coastlines have been altered by land reclamation and development, overfishing, pollution and species invasions (Lotze et al. 2006; Airoldi and Beck, 2007). When coupled with climatic instabilities, localized human perturbations are creating new disturbance regimes which are further accelerating the degradation and decline of coastal ecosystems (Claudet and Fraschetti, 2010). With current delays and limitations on the mitigation of global stressors, it is crucial to identify which factors can maintain ecosystem resilience at local scales (Brown et al. 2013).

Saltmarshes are prime examples of a threatened coastal ecosystem that supports important ecosystem services including maintenance of water quality, provision of habitat, carbon sequestration and control of shoreline erosion (Zedler and Kercher, 2005; Ysebaert et al. 2011; Silliman et al. 2012). Saltmarshes globally have been in steady decline (Lotze et al. 2006), with reductions in Europe by more than 60% (Airoldi and Beck, 2007) and in the US by 50% (Kennish, 2001). Remaining tracts of intact saltmarshes are threatened by a variety of human-related stressors (e.g. Silliman and Bertness, 2004; Goldman Martone and Wasson, 2008; Uhrin and Schellinger, 2011; Lin and Mendelssohn, 2012) including two ubiquitous factors impacting coastal zones worldwide: excess nutrient input, a local scale stressor arising from intensive farming and inadequate wastewater treatment in the watershed, and relative sea level rise (RSLR) accelerated by climate change and amplified in some regions by land subsidence (FitzGerald et al. 2008; Deegan et al. 2012).

Relative sea level rise affects saltmarsh biogeochemistry through a variety of processes. As sea level rises relative to the existing saltmarsh elevation, inundation time of the saltmarsh platform increases. Increased inundation inhibits plant growth due to a combination of mechanisms, including reduced photosynthesis related to stomatal closure, root death due to anoxia, and reduced growth resulting from build-up of toxic compounds in the soil (e.g. hydrogen sulphide) under low redox and anaerobic conditions (DeLaune et al., 1987). In addition, reduced root biomass production hampers the ability of saltmarshes to grow vertically to keep up with RSLR (Morris et al. 2002; DeLaune and Pezeshki, 2003;

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Table 1
Effects of higher nutrient availability on shoot and root biomass, R:S biomass ratio and soil, summarized from enrichment experiments and correlational studies. ↑: increase, ↓: decrease, ↔: no change, NR: not reported.* Trend estimated from reported data.

Reference	Species and study duration	Shoot biomass	Root biomass	R:S biomass ratio	Soil effects	Suggested mechanism
Valiela et al. (1976).	*S. alterniflora* & *S. patens*, 7 months	↑	↓at highest nutrient concentrations	↓*	NR	Some grasses stop producing more roots once N absorption is adequate.
Morris and Bradley (1999).	*S. alterniflora*, 12 years	↓	NR	NR	↓ soil C stores; ↑ sediment accretion	Soil C loss from increased soil respiration; dense shoots trap more sediment.
Boyer et al. (2000).	*S. foliosa*, 2 years	NR	↔	NR	NR	Low N-retention in soil due to sandy substrate or poor conversion rate by microorganisms.
Wigand et al. (2004).	*S. patens*, 2.5 years	↑	↔	↓	NR	Removal of N-limitation allows plants to shift energy to shoot growth, requiring additional P, which can be achieved via stimulation of endomycorrhizal colonization in existing roots, instead of increasing root density.
Tyler et al. (2007).	(a) Invasive hybrid *Spartina* (*S. foliosa x S. alterniflora*), San Francisco Bay, 5 months	↑	↔	↓at edge plots	NR	Root biomass in this eutrophic location likely not N-limited.
	(b) Invasive *S. alterniflora*, Willapa Bay, 5 months	↑	↑ in meadow plots	↓at edge plots	NR	Root biomass in this location is N-limited so N-addition led to increased root growth. However, shoot biomass increased proportionately more than root biomass.
Darby and Turner (2008b).	*S. alterniflora*, *5 months*	↑	↔ when only N added; ↓ when N + P added	↓ when either N or P added	↓ in soil redox potential	N-addition stimulated shoot biomass only; root foraging relaxed when P-availability increased.
Turner et al. (2009).	*S. alterniflora*, *S. patens*, *Disticlis spicata* mixed marsh, 36 years	↑	NR	↓	↔ in soil accumulation rate; organic matter accumulation ↓ in deep soil layer but ↔ in upper layer; ↓ shear vane strength	Increase in carbon loss via denitrification and other coupled processes between C metabolism and N cycle.
Wigand et al. (2009).	*S. alterniflora*, *S. patens* (correlation data)	NR	↓ for *S. patens*	NR	↑ in soil respiration rate for both species	Increased turnover of labile C and N in upper layer of *S. alterniflora* marsh.
Anisfeld and Hill (2011)	*S. alterniflora*, 5 years	↑	↔	↓*	↔ in soil C; ↔ in elevation; slight ↑ in accretion in N + P fertilized plots	Loss of soil C in high nutrient treatment is offset by other processes, e.g. increased gross root production and respiration, leading to no change in belowground primary production.
Nelson and Zavaleta (2012).	*Sarcocornia pacifica*, *Jaumea carnosa*, *Frankenia salina*, and *D. spicata* (perennial), 2 years	↑	↑	↓*	NR	Shoot biomass increase was much larger than root biomass increase; capacity to absorb N remains even in "hypertrophic" environment.
Deegan et al. (2012).	*S. alterniflora* and *S. patens*, 9 years	↑	↓	↓	↓ soil and creek bank stability; conversion of marsh to open water	Faster decomposition of N-rich leaf detritus; increased denitrification and soil

Table 1 (*continued*)

Reference	Species and study duration	Shoot biomass	Root biomass	R:S biomass ratio	Soil effects	Suggested mechanism
						respiration accelerated the decomposition of soil C, leading to higher fine organic matter and water content in soil; reduced root stabilising role coupled with taller shoots with weaker structure and wetter soil caused loss of soil structural integrity.
Fox et al. (2012).	Various, 30 + years	General shift from *S. alterniflora* to *D. spicata* dominated; ↑ where *S. alterniflora* remained	NR	NR	↑ elevation in areas where *D. spicata* became dominant	Nutrient enrichment led to species change from *S. alterniflora* to *D. spicata* dominant; elevation increase in *D. spicata* areas due to higher biomass accumulation, lower decomposition rate and increased mineral sediment trapping.
Langley et al. (2013).	*Schoenoplectus americanus* & *S. paten*, 2 years	↑	↑	↔	NR	No reduction in root foraging even with nutrient addition due to severe N-limitation
Watson et al. (2014).	*S. alterniflora*, *81 days*	↔	↔ in biomass but shift in root morphology from coarse to finer roots	NR	↑ decomposition and sulfide production	Nutrient enrichment under anoxic conditions increased organic matter decomposition resulting in increased sulfide concentration and higher CO_2 flux.

FitzGerald et al. 2008), while increased root death and subsequent loss of root turgor can result in peat collapse which further enhances vertical elevation loss in a detrimental feedback loop (DeLaune et al. 1994). Consequently, increased inundation can disproportionately reduce belowground biomass production, leading to lower root-to-shoot (R:S) biomass ratio (Janousek and Mayo, 2013). Further, reduced shoot production hampers aboveground sediment trapping and accretion, the second mechanism by which saltmarshes maintain their vertical position relative to the tidal frame (Morris et al. 2002). However, these mechanisms may also be counteracted by the hydrological effect of increased sediment deposition rates arising from the higher inundation depths and longer inundation times (Fagherazzi et al. 2012). Increased inundation is just one of several processes related to RSLR that can potentially negatively affect saltmarshes; other effects include higher wave erosion at the saltmarsh edge due to the deepening and enlargement of tidal bays (Fagherazzi et al. 2013) and increased deposition of suspended sediment on the saltmarsh platform reducing sediment supply to the saltmarsh edge and thus reducing the saltmarsh's ability to withstand lateral erosion (Mariotti and Fagherazzi, 2013). The inability of saltmarsh biomass accumulation and sediment accretion to keep pace with increasing sea levels will result in loss of high/mid marshes and conversion of low marshes to mudflats (Schile et al. 2014).

Nutrient enrichment affects estuaries and coastal waters globally (Selman et al. 2008). On one hand, saltmarshes are recognized as important nutrient sinks, mediating the effects of excess nutrients, particularly nitrogen (Craft, 1996). Nitrogen-cycling is

Table 2
Properties of the two experimental soil types. Data are mean and SE over four replicates. For details see methods.

	Low organic matter (LOM)		Medium organic matter (MOM)	
	Mean	S.E.	Mean	S.E.
Bulk density (g/cm^3)	1.3	0.05	0.1	0.07
Organic matter (%)	2.7	0.49	10.0	0.96
Macro-organic matter (%)	0.6	0.22	4.7	2.35
Sand (%)	80.3	2.06	78.2	3.72
Silt (%)	17.9	1.89	19.8	3.40
Clay (%)	1.9	0.20	2.0	0.32

Table 3
Number of pots with alive or dead plants in October 2013 and number of missing pots due to a storm just after the set-up. Percent survival was calculated as number of pots with alive plants divided by number of available pots at start (four replicates minus missing pots).

Plant source	Nutrient	Inundation level	Soil	No. alive	No. dead	No. missing	Survival (%)
Bellocchio	Low	Low	LOM	4	–	–	100
			MOM	4	–	–	100
		High	LOM	4	–	–	100
			MOM	4	–	–	100
	High	Low	LOM	3	–	1	100
			MOM	4	–	–	100
		High	LOM	4	–	–	100
			MOM	4	–	–	100
Vallona	Low	Low	LOM	4	–	–	100
			MOM	3	1	–	75
		High	LOM	–	3	1	0
			MOM	2	1	1	67
	High	Low	LOM	3	1	–	75
			MOM	4	–	–	100
		High	LOM	2	2	–	50
			MOM	2	1	1	67

Table 4
PERMANOVA analysis of the effects of inundation level (In, low vs. high), nutrient level (Nu, low vs. high) and soil type (So, LOM vs. MOM) on nine response variables for *Spartina* plants sourced from Bellocchio. Here the simplified model is shown, where highly non-significant interactions terms ($p > 0.25$) were sequentially removed following Underwood (1997) and Anderson et al. (2008) (see Appendix S2 for the full models). Significant p values ($p < 0.05$) are in bold, while marginally significant values ($0.05 < p < 0.1$) are in italics.

	No. of shoots				No. of flowers			Shoot biomass		
Source	df	MS	F	P	MS	F	P	MS	F	P
In	1	29,482	18.55	**0.0001**	21.33	10.19	**0.004**	260.4	26.67	**0.0001**
Nu	1	7067	4.45	**0.046**	3.41	1.63	0.221	55.4	5.67	**0.023**
So	1	1987	1.25	0.281	0.65	0.31	0.591	6.9	0.71	0.411
In x Nu	1	6552	4.12	**0.049**	4.81	2.3	0.149	62.3	6.38	**0.016**
In x So	1	4408	2.77	0.114	15.41	7.36	**0.012**	43.7	4.48	**0.047**
Nu x So	1	3414	2.15	0.157	15.41	7.36	**0.013**	41.4	4.24	*0.051*
In x Nu x So	1	4137	2.6	0.125	18.25	8.72	**0.007**	34.2	3.5	*0.071*
Residual	23	1589			2.09			9.8		
Total	30									

	Root biomass				Mean tallest shoot height			Root: shoot biomass ratio		
Source	df	MS	F	P	MS	F	P	MS	F	P
In	1	84	27.86	**0.0002**	70.8	7.33	**0.011**	17.4	6.61	**0.006**
Nu	1	5.1	1.71	0.21	0.1	0.01	0.922	2.3	0.86	0.4
So	1	0.2	0.07	0.792	2.3	0.23	0.633	4.1	1.56	0.239
In x Nu	1	27.5	9.14	**0.006**	37.7	3.91	*0.058*			
In x So	1	9.6	3.19	*0.085*	18.4	1.9	0.181	5.9	2.26	0.15
Nu x So	1	19.8	6.58	**0.015**						
In x Nu x So	1	12.1	4.02	*0.058*						
Residual	23	3								
Pooled	25[a]				9.7			2.6		
Total	30									

	Soil loss				Soil redox		
Source	df	MS	F	P	MS	F	P
In	1	0.79808	1.5076	0.2308	0.0028525	1.3091	0.2621
Nu	1	0.72242	1.3647	0.2583	0.0018238	0.83698	0.371
So	1	0.33743	0.63742	0.4441	0.005326	2.4442	0.1261
In x So	1	3.1992	6.0434	**0.0224**			
Pooled	26[b]	0.53652					
Total	30						

[a] 25 for mean tallest shoot height and 26 for root: shoot biomass.
[b] 26 for soil loss and 27 for soil redox.

generally so efficient in saltmarshes that marsh ecosystems tend to be nitrogen-limited (Valiela et al. 1978; Mendelssohn, 1979; Kiehl et al., 1997; Boyer et al. 2001; Crain, 2007). Excess nitrates are removed by assimilation into primary production, denitrification (Mitsch and Gosselink, 2000; Wigand et al. 2009), and to a much lower extent by bacterially-mediated anaerobic ammonia oxidation (Koop-Jakobsen and Giblin, 2009). On the other hand, concerns are being raised that increased nutrient availability to saltmarshes could be facilitating saltmarsh loss (Deegan et al. 2012). Some evidence shows that high nitrogen availability can suppress belowground biomass production and accumulation and perhaps increase organic matter decomposition, resulting in loss of soil stability, increased soil erosion and saltmarsh habitat loss (Turner et al. 2009; Turner, 2010; Deegan et al. 2012; Watson et al. 2014). With respect to the effect of nutrient enrichment on root biomass, short- and long-term mesocosm and field experiments have produced mixed responses in saltmarsh vegetation (Table 1). Nutrient enrichment reduced root biomass growth in most studies, while increased or unaffected growth has also been reported. Furthermore, the direct and indirect effects of elevated nutrient availability on saltmarsh soil has also been mixed (Table 4 and review by Morris et al., 2013). These mixed results in vegetation responses most likely relate to differences in the magnitude of nutrient enrichment (Valiela et al., 1976) and species types (e.g. Ravit et al. 2007; Langley et al., 2013).

Since nutrient cycling occurs in a large part in saltmarsh sediments, the local soil conditions could significantly modify the effects of nutrient enrichment. Saltmarshes can be found on a variety of soils, ranging from mineral-dominated to organic-dominated (Mitsch and Gosselink, 2000). Studies comparing mature saltmarshes with recently formed saltmarshes (both natural and man-made) report that the soil bulk density tends to decrease while particulate organic matter, soil carbon and nitrogen content increase as saltmarshes mature (Craft et al., 1988; Osgood and Zieman, 1993; Craft, 2000; Havens et al., 2002; Edwards and Proffitt, 2003). As far as we are aware, no studies have empirically addressed how saltmarshes of different soil types and with different nutrient availability respond to increased inundation.

We conducted a field experiment to investigate the response of low intertidal saltmarsh growth to increased inundation in different soil types and nutrient availabilities. We chose the nutrient levels based on realistic nutrient conditions of lagoons in the study region while the high inundation level simulated a sea level increase of 10 cm which has been estimated to occur close to year 2020 based on sea level scenarios developed for the Italian North Adriatic coastline (THESEUS, 2009). We chose two soil types that differed in the amount of organic matter content, reflecting respectively emergent or restored marshes with lower organic content and mature marshes with higher organic content. We predicted that (i) saltmarsh vegetation will be negatively affected by an increase in inundation as a result of a 10 cm simulated increase in water level relative to current sea level, (ii) low nutrient conditions will help to maintain greater root biomass and R:S ratios thereby mitigating the negative effects of higher inundation, and

(iii) the effects of higher nutrients on root growth will be dampened in soils with higher organic content due to its ability to support higher rates of denitrification. We focused on *Spartina maritima* (hereby referred to as *Spartina*) as this is the most relevant foundation species in the low intertidal saltmarsh vegetation in the study region.

2. Methods

2.1. Study area

The Po Delta is located in the northern Adriatic and is bordered by the Venice Lagoon to the north and sandy beaches of the Romagna to the south. The delta and adjoining coasts are characterised by low relief and shallow coastal wetlands and beaches and experience an average tidal range of 0.8 m while tides up to >1.8 m can occur during extreme storm surges. A large part of the delta lies below sea level and is defended by numerous coastal protection structures (Cencini, 1998). Saltmarshes in this region have one of the longest history of degradation and conversion (Airoldi and Beck, 2007). While direct conversion is now restricted, saltmarshes are faced with other continuing human impacts, including excessive nutrient input from intensive farming, industry and dense urban centres in the watershed (Nespoli, 1988) and RSLR driven both by global increasing sea level and localised land subsidence (Cencini, 1998). Pockets of extensive coastal saltmarshes remain, but growth dynamics and basic ecological processes are poorly known (Scarton et al., 2002).

Spartina plugs used in the mesocosm experiment were collected from two lagoons; Vallona Lagoon and Bellocchio Lagoon. Vallona Lagoon (45°01′40.8″N, 12°23′01.4″E) is located in the current-day Po Delta. The lagoon was created unintentionally in the 1970's when previously drained farmland was re-flooded by seawater due to severe land subsidence. Like the rest of the coast, Vallona Lagoon experiences a semi-diurnal micro-tidal regime (Consorzio di Bonifica Delta del Po, 2013). It is used privately for shellfish aquaculture and seasonal hunting, and has sluice gates that are actively managed to maximize water exchange and maintain good water quality (Fondazione Ca' Vendramin, 2010), such that compared to other lagoons in the Veneto region, it has a moderate to low level of nitrate (Appendix S1). Bellocchio Lagoon (44°37′39.8″N, 12°15′55.8″E) is located about 50 km south of Vallona Lagoon in the fossil Po Delta which is no longer part of the active delta. The Bellocchio Lagoon is a back-barrier lagoon behind an active sand dune and is connected to the sea by a channel that is maintained by periodic dredging. It is one of few lagoons with low nitrate levels in the region (Lo et al. unpublished data). Both lagoons are small and sheltered by natural or man-made barriers, but are freely influenced by tides. The low intertidal saltmarsh assemblage is similar at both lagoons, predominantly comprising of mono-specific tussocks of *Spartina* interspersed with *Salicornia veneta* and bare soil at an elevation of 0.1–0.3 m above mean sea level (a.m.s.l.). *Spartina* is a perennial grass growing on a variety of substrate types ranging from silt to sand and peat. In the Vallona Lagoon, *Spartina* has created patches of organic-rich soil in the parent substrate, which is predominantly mineral soil. The same species also dominate the remaining saltmarshes in the region. Information on the geomorphology, hydrology and environmental characteristics of the two lagoons of the region can be found in Villatoro et al. (2014) and Fondazione Ca' Vendramin (2010).

Fig. 1. Layout of the experiment at Vallona Lagoon. Pots were arranged in two rows to different inundation levels (low vs. high). Each row contained four replicates for each combination of two nutrient treatments (high vs. low conditions) and two soil types (low organic matter, LOM vs. medium organic matter, MOM) for a total of 32 pots per row.

2.2. Experimental design and setup

The mesocosm experiment was carried out in Vallona with plugs from both lagoons. The mesocosm was not replicated in Bellocchio Lagoon to avoid impacts to the small and fragile natural ecosystems, which are protected as a Natura 2000 Site of Community Interest. In July 2013, we simulated eight scenarios with orthogonal combinations of two inundation levels (low vs. high inundation), two nutrient levels (low vs. high nutrient conditions) and two soil types (low organic matter LOM vs. medium organic matter MOM). Each scenario was replicated eight times ($n = 8$), for a total of 64 units. We planted half the units with *Spartina* plugs from Vallona and the other half with plugs from Bellocchio. Plugs with shoots and roots were collected from the edge of *Spartina* colonies and were thinned with scissors to the soil surface (Pennings et al. 2005) until five shoots of 10–15 cm height remained. We ran the experiment for three months over the main growing and flowering period, i.e. July–September.

Experimental units consisted of round PVC-pipes (16 cm diameter, 40 cm height), hereby referred to as a pot. Each pot comprised an open top and closed bottom lid. We drilled eight holes in each lid to facilitate vertical water movement and lined the bottom with a nylon mesh to prevent mixing of the soil within the pots with the surrounding native soil. Each pot was pushed into the substrate until the top of the pot reached the designated height; we then secured the pots with cable ties to three 1.5 m poles hammered into the surrounding substrate to prevent movement during storms.

The pots were equally distributed in two parallel rows 1.5 m apart (Fig. 1). We manipulated inundation time by varying the height of the pots. These pots are a modification of the “marsh organ” technique (Morris, 2007) in that individual pots are not linked to other pots and have a bottom cap and thus fixed volume of soil. The “low” inundation level was defined as the average tidal level at which *Spartina* is currently growing at the experimental site (0.1 a.m.s.l.) while the “high” inundation was set 10 cm lower (0 a.m.s.l.). Based on sea level data from the ISPRA regional monitoring programme (http://www.venezia.isprambiente.it) from the northern Adriatic as well as tidal level data loggers deployed at Bellocchio Lagoon, a decrease in elevation of 10 cm corresponds approximately with a 20% increase in inundation time.

Soil type and nutrient levels were imposed orthogonally at random to each of the inundation regimes (Fig. 1). We collected the two soil types from within a 200 m radius of the experimental site in the intertidal zone. The LOM substrate was taken from unvegetated areas while the MOM substrate from vegetated areas patchily interspersed within the intertidal zone. At the start of the experiment, we collected four replicate soil samples from each of the two soils types for analyses of macro-organic matter content, organic matter content (using the Loss on Ignition (LOI) method) and soil bulk density. For each replicate sample, we washed 30 ml of sample over a 2 mm mesh diameter sieve to separate out the macro-organic matter which was dried at 70 °C and weighed. Then, we weighed and dried another 20 ml of sample at 70 °C to constant weight to obtain the bulk density. We ignited a sub-sample from the bulk density analysis at 450 °C for 4 h to estimate the organic matter ash free dry weight (Curado et al. 2013). The LOM treatment consisted of sediment with low organic content (2.7% ± 0.49) and high bulk density (1.3 ± 0.05 g/cm^3) while the MOM treatment had higher organic content (10.0% ± 0.96 g/cm^3) and low bulk density (0.1 ± 0.07 g/cm^3) (Table 2). Both soil types were predominantly fine sand (125–250 μm) with a total sand content (62.5 μm – 1.6 mm) of approximately 80%. After filling with the assigned soil type, the pots were left for two weeks to allow for soil settlement before the experiment. At the start of the experiment, each plot was planted with one plug and re-filled to the top with the assigned soil type.

We selected nutrient treatment levels to reflect realistic nitrate concentrations in coastal lagoons monitored by the Veneto Regional Environmental Protection Agency (see Appendix S1 for details). The existing nitrate level of Vallona Lagoon was chosen as the low nutrient scenario while the high nutrient level was based on the nearby Caorle Lagoon (Figure S1.1) which records some of the highest nitrate concentrations in the region due to a combination of factors, including reduced water exchange and land-based runoff (Fondazione Ca' Vendramin, 2010). We achieved the high nutrient level with the slow-release fertilizer Osmocote® Exact Standard NPK. Pre-tests indicated that a dose of 45 g/pot achieved a soil porewater nitrate-N concentration of approximately 1.5 mg/L (Appendix S1, Figure S1.2).

2.3. Measured response variables

At the start of the experiment, we set aside ten reference *Spartina* plugs from each donor site for analysis of live shoot and root biomass. We clipped the live shoots at the soil surface and washed root clumps carefully over a 1 mm sieve to remove all sand and silt while large organic and inorganic debris were removed by hand. Live roots were distinguished from dead roots and other dead organic matter by colour, texture and density (Darby and Turner, 2008). Live dry biomass for roots and shoots were obtained after drying separately at 70 °C to constant weights, and then weighed to the nearest 0.1 g.

Four replicates were lost immediately after the set-up as a strong storm washed away the transplanted plants. Those pots were removed from the calculations; therefore, the number of available replicated pots varied between three and four. At the end of the experiment, we estimated percentage survival of plants per treatment as number of pots with living *Spartina* over number of available pots. In pots with remaining *Spartina* (live and dead) at the end of the experiment, we estimated soil loss by measuring the distance of the soil surface within each pot relative to the top of the pot at four haphazard positions. We took four redox readings from each pot for the first 2 mm surface soil with a handheld probe (Hanna Instruments HI 8314 with redox probe HI 3230).

All live plants were then extricated carefully from the pots and brought to the lab for the following measures: number of live shoots, height of the five tallest live shoots and number of flowers per pot. After these measures were taken, we analysed the samples for live shoot and root biomass following the procedure used for the reference *Spartina* plugs at the start of the experiment. We obtained the R:S ratio by dividing live root dry biomass by live shoot dry biomass. We analysed representative samples of root and shoot biomass from each pot for elemental carbon (C) and nitrogen (N) composition (Organic Elemental Analyzer Flash 2000, Interscience). Dead plants were scored as having zero root and shoot biomass and zero number of shoots and flowers, while tallest live shoot height, C and N content and R:S ratio were considered as missing values.

2.4. Statistical analysis

While all *Spartina* from Bellocchio survived to the end of the experiment and showed changes in tissue N content consistent with the nutrient manipulation, the plugs from Vallona experienced high mortality, leading to lack of sufficient numbers of live plants for statistically meaningful measurements (see Results). Therefore, plants from Vallona were not further analysed.

The responses of *Spartina* from Bellocchio to inundation in different nutrient environments and soil types were analysed using permutational ANOVAs with three fixed orthogonal factors: inundation (low vs. high), nutrient level (low vs. high), and soil type (LOM vs. MOM). The variables analysed were: number of shoots, number of flowers, mean height of tallest live shoots, live shoot and root biomass, R:S biomass ratio, shoot and root C:N ratio, and soil loss. We used the statistical procedure PERMANOVA in PRIMER (Anderson et al., 2008) to partition the variability and obtain F-statistics on matrices of Euclidean distances calculated from the original raw data. *P*-values were calculated using 9999 random permutations of the appropriate exchangeable units and Type III sums of squares under the reduced model. All analyses began with a full model, with all possible interactions included; a final simplified model was selected by sequentially dropping highly non-significant interaction terms ($P > 0.25$) following Underwood (1996) and Anderson et al. (2008). Statistical results for the full models are presented in Appendix S2. We used permutational ANOVA rather than the classic ANOVA test due to ease of use with the slight unbalanced number of replicates (3–4) arising from one missing pot. All variables were tested for homogeneity of dispersion between groups with the PERMDISP function in PERMANOVA. No significant dispersion was detected.

3. Results

All plants from Bellocchio survived to the end of the experiment (Table 3). Plugs increased from the initial five shoots to an average of 44 ± 9.6 shoots per pot (Fig. 2), and produced on average 2 ± 0.4 flowers per pot. The CHN analysis revealed percentage N and C: N ratios of the roots to be significantly higher and lower, respectively, in the high than low nutrient treatments; this was consistent with the experimental nutrient manipulation (see Appendix S1 for details). Thirty-three percent of plants from Vallona died (Table 3), with the highest mortality in the increased inundation treatments in LOM soils. The surviving plants from Vallona did not grow nor produce flowers (Appendix S3) and tissue N content did not seem to show differences in nutrient uptake between treatments (Appendix S1). Because of insufficient numbers of live plants for statistically meaningful replicated measurements, and no evidence of different nutrient uptake between treatments, plants from Vallona were excluded from further analyses, and only data from Bellocchio are presented. The data collected from the surviving

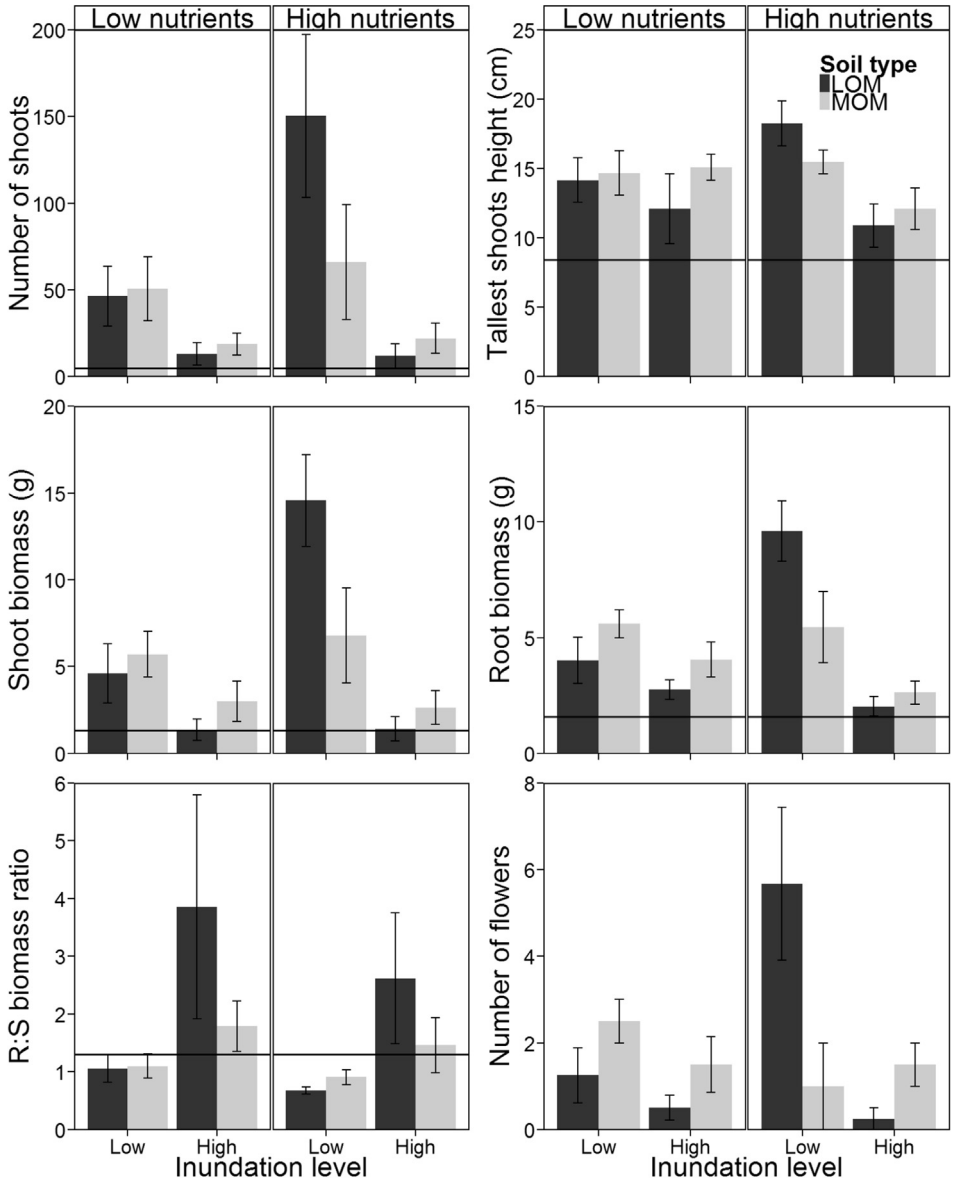

Fig. 2. Number of shoots, shoot height, shoot biomass, root biomass, R:S biomass ratio and number of flowers of *Spartina* from Bellocchio in response to different inundation levels (low vs. high), nutrient levels (low vs. high), and soil type (low organic matter, LOM vs. medium organic matter, MOM). Horizontal lines indicate baseline values measured from the reference *Spartina* at the start of experiment. Data are average amounts per pot ($n = 3 - 4$, ±1 SE).

plants of Vallona are reported in Appendix S3 for completeness of results.

The effects of increased inundation on *Spartina* varied with both nutrient levels and soil types (Fig. 2). High inundation resulted in lower mean shoot and root biomass and number of flowers; the largest decreases in these plant measures compared to low inundation occurred in the high nutrient with LOM soil treatments (significant or marginally significant inundation × nutrient × soil interactions, Table 4). Number of shoots and mean tallest shoot heights also decreased between low and high inundation levels; these differences were largest at high nutrient conditions (significant or marginally significant inundation × nutrient interactions, Table 4), while soil type did not lead to significant effects (Fig. 2, Table 4). Treatments did not result in significant differences in soil redox potential (Table 4).

High inundation disproportionately affected root and shoot biomass resulting in significant effects on R:S ratios. Increased inundation decreased biomass growth in both shoots and roots, but the effects were more severe for shoots, leading to overall higher R:S ratios under high inundation conditions compared to low inundation treatments. Lower nutrient condition led to higher R:S ratios across both inundation levels and soil types, with the highest R:S ratio found in the low nutrient combined to LOM soil treatment, but these trends were not significant (Table 4). MOM pots lost on average 1.6 ± 0.35 cm and 2.6 ± 0.29 cm of soil relative to the top of the pots in low and high inundation treatments respectively, while no differences with inundation were observed for LOM pots, which lost on average 1.9 ± 0.13 cm of soil (significant inundation × soil interaction, Table 4). No differences in soil loss were observed between nutrient treatments.

4. Discussion

The simulated inundation increase had a strong negative effect

on most *Spartina* plant growth parameters, particularly in LOM soils. The R:S biomass ratio increase in the higher inundation treatments reflected a greater tolerance of roots than shoots to increased inundation, rather than an increase in root biomass. These negative effects of increased inundation on *Spartina* biomass production corroborated with results of other mesocosm experiments (Voss et al., 2012; Watson et al. 2014). However, our plants were more sensitive than reported in experiments with the same species from other regions (Adams and Bate (1995) in South Africa; Castillo et al. (2000) in Odiel, Spain). We saw similar increased sensitivity in a recent large-scale, marsh-organ experiment replicated at several locations around Europe including the north Adriatic sea (van Belzen et al. unpublished data). This distinct negative response might be related to the microtidal nature of the Mediterranean system where *Spartina* grows in a relatively narrow range of elevations (Ibáñez et al. 2000), such that it may be less adapted to large sustained changes in inundation. It could also indicate that the current elevation of the *Spartina* is already at or below the elevation for optimal growth; theoretical models and empirical data predict that saltmarshes at elevations below the optimum level are more vulnerable to increasing relative sea level compared to systems that are located at higher than optimal elevations (Morris et al. 2002; Kirwan and Guntenspergen, 2012).

Responses to treatments differed between plants from different source locations; increased inundation resulted in particularly high mortality for plants sourced from Vallona Lagoon. We exclude that this particularly negative response was related to any experimental procedure, as it was not observed for plants from Bellocchio, which were subjected to the greatest potential stress due to transportation to the Vallona Lagoon study site. The surviving plants from Vallona had lower growth rates, biomass and reproductive potential than plants from Bellocchio, and did not seem to respond to the nutrient addition. One possible hypothesis is that the Vallona strain might be a distinct cryptic species previously undescribed in our study region. Preliminary results from a broad-scale genetic study, conducted one year after the conclusion of this experiment and still in progress, indicate the presence of the alien *Spartina anglica* in our study region, including the Vallona lagoon (Wong et al., unpublished data). This invasive hybrid has hitherto been un-documented in this region likely due to difficulties in identification via morphology and similarities in habitat with the native *S. maritima*. We are now trying to clarify the exact distribution and abundance of the invasive species in our region using molecular tools. Whether the two strains are different cryptic species or discrete phenotypes, this result suggests that site-specific responses to increased inundation at the foreshore of saltmarshes can be extremely variable, and should be incorporated into regional conservation planning.

The effects of increased inundation on *Spartina* were modified by nutrient conditions and soil type. Our hypothesis that low nutrient conditions would mitigate the negative effects of increased inundation by maintaining greater R:S ratio was only partially supported by our results. On average, biomass differences between inundation treatments were larger at high than low nutrient conditions, particularly in LOM soils. This suggests that saltmarshes in high nutrient environments will face larger decreases in biomass with increased inundation as a result of accelerated sea level rise than saltmarshes in low nutrient environments, and that this will be particularly relevant for saltmarshes in LOM soils that are often associated with younger saltmarshes. However, the expected benefits of low nutrients on root biomass in plants experiencing higher inundation were less than predicted, and did not result in significant improvements in root growth. We hypothesize that the distinct large negative effects of increased inundation may have masked nutrient effects. A study that manipulated inundation, nutrients and CO_2 in tandem (Langley et al. 2013) also found that inundation was the dominant stressor on biomass production and the R:S ratio, although nutrient addition increased growth and conferred greater survival to saltmarsh plants under moderately increased flooding conditions. In contrast, another mesocosm experiment manipulating inundation, nutrient availability and precipitation with *Spartina alterniflora L.* found no effect of nutrient increase on root and shoot biomass, although root morphology shifted from coarse to finer roots with increased nutrients (Watson et al. 2014). The variability of saltmarsh root growth to increased nutrients suggests that responses are likely to be case specific, dependent upon soil type, species and magnitude of enrichment (see also Table 1).

Smaller biomass differences between nutrient treatments in MOM soil (compared to LOM soil) support our second hypothesis that MOM soils can dampen the effect of increased nutrients on root growth. MOM soil is more efficient in removing additional inorganic nitrogen species compared to LOM soil via a combination of denitrification and the coupled nitrification-denitrification of ammonia. Under anaerobic soil conditions, denitrifying bacterial activities result in the removal of nitrates and consumption of organic carbon with production of dissolved organic carbon (Ingersoll and Baker, 1998). In coupled nitrification-denitrification, ammonia is first converted to nitrite then to nitrate by nitrifying bacteria followed by denitrification in a coupled process (An and Joye, 2001). Coupled nitrification-denitrification is likely to be the predominant pathway for nitrogen loss in saltmarsh platforms (Koop-Jakobsen and Giblin, 2010) although other biotic and abiotic pathways also play a role (Burgin and Hamilton 2007), such as the physical flushing of nutrients from the soil porewater. The latter mechanism tends to be more important in well-drained sandy substrates than in cohesive organic-rich substrates (Thompson et al. 1995). Thus, although organic soils supporting mature saltmarshes have large amounts of nitrogen locked up in the soil particulate organic matter compared to young saltmarshes (Craft et al. 1988; Langis et al., 1991), they also support high rates of denitrification resulting from low oxygen levels and the longer retention time of dissolved nutrients in soil porewater (Thompson et al. 1995).

Measuring differences in soil erosion as a consequence of inundation and nutrient concentrations in the environment was beyond the scope of this manuscript, and it would have required specific dedicated experiments using undisturbed soil cores. Nevertheless, the largest loss of soil observed in pots with MOM soils under increased inundation is consistent with results from other work suggesting that large organic detritus reduces soil cohesiveness (Feagin et al. 2009). Additionally, a high nutrient availability can increase rates of paired sulfate reduction and organic matter decomposition (Watson et al. 2014) which could have further reduced soil volume, leading to soil elevation loss within the pots. These preliminary observations suggest that useful future work could further explore the links between nutrient concentrations, vegetation growth and soil erodibility, and how these could be modified under different inundation regimes.

5. Conclusion and implications for management

Complex interactions between nutrients and various natural and anthropogenic factors are known from many marine ecosystems (e.g. Breitburg et al. 2009; Falkenberg et al. 2013; Strain et al. 2014; Thrush et al. 2014). This first work on the effect of nutrient levels and soil type in modifying the effect of increased inundation on saltmarsh vegetation reveals empirical evidence that can assist in the implementation of ecosystem-based management for saltmarshes under accelerated sea level rise. Even though increasing

inundation as a result of accelerated sea level rise will be gradual and likely result in longer term vegetation growth and sedimentation feedbacks, our results provide an indication of the expected direction of change under the different simulated scenarios.

Overall, our results supported the hypothesis that increased inundation would have a negative effect on *Spartina* survival and biomass production and that the reduction in productivity would be greatest in LOM soils representing young saltmarshes, particularly at high nutrient levels. Newly created saltmarshes may therefore be effective nitrogen sinks to mediate excess nutrients arising from eutrophication; however, this service will be severely hampered if the saltmarsh elevation is unable to keep up vertically with sea level rise, or if saltmarsh zones are unable to migrate to higher elevations, e.g. due to the presence of hard structures (Donnelly and Bertness, 2001) or lack of upland marshes as in saltmarsh islands (Schile et al. 2014). Finally, the effect of nutrient enrichment (and conversely nutrient management) on *Spartina* vegetation is likely to be very site and condition specific. Managers should therefore be careful not to generalize effects between different saltmarshes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.marenvres.2014.12.003.

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